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<b>(21) International Application Number:</b> PCT/US91/06727 <b>(22) International Filing Date:</b> 20 September 1991 (20.09.91) <b>(30) Priority data:</b> 589,933 28 September 1990 (28.09.90) US <b>(60) Parent Application or Grant</b> <b>(63) Related by Continuation</b> US 589,933 (CIP) Filed on 28 September 1990 (28.09.90) <b>(71) Applicant (for all designated States except US):</b> THE UPJOHN COMPANY [US/US]; 301 Henrietta Street, Kalamazoo, MI 49001 (US).		<b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only) :</b> GREENBERG, Barry, D. [US/US]; 5140 Chickadee Drive, Kalamazoo, MI 49002 (US). <b>(74) Agent:</b> DELUCA, Mark; Corporate Patents & Trademarks, The Upjohn Company, Kalamazoo, MI 49001 (US). <b>(81) Designated States:</b> AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH (European patent), CI (OAPI patent), CM (OAPI patent), CS, DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GA (OAPI patent), GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU (European patent), MC, MG, ML (OAPI patent), MN, MR (OAPI patent), MW, NL (European patent), NO, PL, RO, SD, SE (European patent), SN (OAPI patent), SU*, TD (OAPI patent), TG (OAPI patent), US.  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>

**(54) Title:** TRANSGENIC ANIMALS WITH ALZHEIMER'S AMYLOID PRECURSOR GENE

**(57) Abstract**

A transgenic rodent useful for studying Alzheimer's disease having a transgene comprising a mammalian metallothionein I (MtI) promoter operably linked to a nucleotide sequence encoding Alzheimer amyloid precursor protein (AAP protein) operably linked to a mammalian growth (GH) hormone 3'-untranslated region is disclosed.

# + DESIGNATIONS OF "SU"

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TRANSGENIC ANIMALS WITH ALZHEIMER'S AMYLOID PRECURSOR GENE  
FIELD OF THE INVENTION

The present invention relates to transgenic animals useful as models for studying Alzheimer's disease and useful for identifying compounds for treating Alzheimer's disease.

5 BACKGROUND OF THE INVENTION

Alzheimer's disease (AD) is the most common cause of dementia in late life. AD results in a progressive loss of intellectual function characterized by progressive impairments in memory, language, visuospatial skills and behavior. Those afflicted with AD eventually become unable to speak or think or take care of themselves. AD is a terminal disorder, but patients generally die  
10 of some complication that afflicts bedridden patients. It is estimated that in the United States, from 1.5 to two million people suffer from this degenerative disorder of the central nervous system.

What causes AD and how its characteristic changes are brought about are not known. There is no known treatment or cure for AD. The diagnosis of AD can only be inferred during the patient's lifetime since no unique pattern of behavioral abnormalities has been established and  
15 there is no satisfactory laboratory test short of a brain biopsy. An autopsy, however, shows highly characteristic pathologic changes in the brain.

The clinical manifestations of AD are the result of a degeneration of neurons, particularly in regions essential for memory and cognition, or thought processes. There is a loss of neurons located in the basal forebrain cholinergic complex, several monoaminergic brainstem nuclei,  
20 amygdala, hippocampus and neocortex. There is a significant loss of neurons in certain more primitive regions at the base of the brain, with consequent reduction in the amount of the neurotransmitters, notably acetylcholine, normally released from the terminals of those neurons in higher brain centers.

AD is associated with abnormal protein structures. The three major pathologic signs of  
25 AD are neurofibrillary tangles within neurons, amyloid surrounding and invading cerebral blood vessels and amyloid-rich plaques proximal degenerating nerve terminals. Each of these signs reflects an accumulation of proteinaceous structures not normally found in the brain.

Neurofibrillary tangles result from accumulation of proteinaceous deposits which form abnormal fibers within the perikaryon of neurons. These accumulations of twisted filaments and  
30 other abnormal structures are found within neuronal cell bodies and contribute to the degeneration of nerve cell processes.

In addition to neurofibrillary tangles, a central feature of the pathology of AD is the presence of deposits of amyloid within plaques and around blood vessels. The major diagnostic lesion of AD is the deposits of abnormal amyloid proteins in intracellular and extracellular  
35 locations. The cellular dysfunction and death that eventually result from these deposits are common consequences of diseases termed "amyloidosis", which are characterized by the deposition

of abnormal fibrillar proteins in these extracellular and intracellular spaces.

The term "amyloid" is applied to pathological accumulations within tissues of a protein-rich mass notable mainly for its staining properties: when amyloid is stained with a dye called Congo red and viewed under polarized light, it emits a greenish yellow glow, and under polarized light, a red/green birefringence. Some amyloid is seen in the brain of most old people and in other organs, such as the liver and kidney, of people with certain chronic diseases. Abundant cerebral amyloid is, however, always associated with AD, where it is seen as deposits in and adjacent to blood vessels and as a components of neuritic plaques. The abnormal proteins of the neurofibrillary tangles also can exhibit the staining properties of amyloid.

The neuritic (or senile) plaque is the pathological structure whose presence signals AD to the neuropathologist. Plaques are usually most abundant in the cerebral cortex and hippocampus and in the amygdala, a nucleus of cells near the hippocampus that seems to be particularly damaged in the disease. Within each region the plaques are localized in areas containing the axonal terminals of neurons rather than their cell bodies. The consistent evidence that the fibrillar deposits in plaques and cerebral vessels are amyloid fibers and that the paired helical filaments in tangles are twisted,  $\beta$ -pleated sheet fibrils, have led to the conclusion that AD is a form of cerebral amyloidosis. This signifies that the above lesions may be directly or indirectly responsible for neuronal cell death and represent an important stage of the pathogenetic process leading to AD.

Biochemical studies have revealed that the plaque core protein in AD is formed from a 4500-dalton protein. The protein is referred to as either amyloid A<sub>4</sub>, or as the  $\beta$ -protein. The full-length protein consists of only 42 to 43 residues. The discovery of  $\beta$ -protein from amyloid-laden cerebral vessels of patients with AD has provided a means to begin deciphering the pathogenesis of AD.

Considerable evidence has accumulated that most amyloid fibril proteins are formed from precursor proteins by proteolytic cleavage to produce  $\beta$ -pleated sheet fibrils and that the precursor proteins have an abnormal sequence or amino acid substitution. Based on these precedents, one would expect the amyloid fibril  $\beta$ -protein of cerebrovascular amyloid, having a maximum of 43 amino acids, to be formed by proteolytic cleavage of a putative abnormal  $\beta$ -protein precursor. Proteolysis of the precursor to form  $\beta$ -protein is accepted; however, despite precedent, no evidence for an abnormal  $\beta$ -protein precursor in AD has thus far been demonstrated. Cloning and cDNA sequencing have indicated that the self-aggregating amyloid protein of AD is encoded as part of one of three larger precursor protein genes. Each protein is referred to as the Alzheimer's Amyloid Precursor Protein (AAP Protein). The respective proteins have 695 residues (AAP<sub>695</sub>), 751 residues (AAP<sub>751</sub>), and 770 residues (AAP<sub>770</sub>). The AAP proteins are encoded by a unique gene on chromosome 21. The various mRNAs are generated by alternative splicing of this gene's primary transcript.

An interesting observation is that the brains of Down's patients who grow to adulthood degenerate in much the same way as those of Alzheimer's patients. Bio-chemical studies have revealed that the plaque core protein in both Alzheimer's disease and Down's syndrome is the identical  $\beta$ -protein. Since the gene encoding AAP protein resides on chromosome 21, overexpression of all AAP may affect the associated amyloidosis. Thus, any treatment to slow or prevent the progression of AD may be useful in the treatment of adult Down's patients.

Presently, the only animal models available to study AD and screen compounds which may be useful for treatment of AD are aged primates which exhibit age-associated memory deficits. These animals display structural/chemical changes in the brain similar to those found in aged humans, particularly those suffering AD. However, the usefulness of these animals is limited and a better animal model is desired.

Among the uses foreseen for a better AD animal model is the ability to use such a model to screen compounds useful in prevent, slow or reverse the accumulation of amyloid in the brain. Identification of such compounds could provide potential therapeutics for AD.

The present invention provides a transgenic animal useful as a model to study the accumulation of amyloid in brain tissue. Furthermore, the present invention relates to a transgenic animal useful in the identification of compounds which can prevent, slow or reverse the accumulation of amyloid in the brain. The present invention provides a transgenic animal useful in the discovery of drugs for the treatment AD and for the prevention of brain tissue degeneration in adults with Down's syndrome. According to the present invention, a transgenic animal is provided which displays tissue-specific overexpression of a gene encoding AAP protein in the regions of the brain where amyloid deposits are commonly found in patients with AD. Thus, amyloid deposits are produced in the transgenic animal models of the present invention in the same pattern as those occurring in AD patients. The transgenic animals of the present invention therefore provide an *in vivo* model which possesses a physical condition that closely resembles a pathological condition of patients afflicted with AD.

#### INFORMATION DISCLOSURE

Swanson, et al., "Novel developmental specificity in the nervous system of transgenic animals expressing growth hormone fusion genes", Nature, Vol. 317, 26 September 1985, pp. 363-366, report that transgenic animals expressing rat-growth hormone (rGH) under control of the mouse metallothionein I (mMtl) promoter express such proteins in a tissue specific pattern in neuronal cells. Similar experiments were performed using transgenes containing mMtl promoter controlling expression of a human growth hormone gene. Localized expression in neuronal tissue of transgenic mice was observed. It is noted that neither metallothionein nor growth hormone are locally expressed in the neuronal cells which express these fusion genes. Fusion proteins containing other structural genes under the control of the mMtl promoter did not exhibit similar

pattern of expression.

Russo, A. F., et al., "Neuronal Expression of Chimeric Genes in Transgenic Mice", *Neuron*, Vol. 1, June, 1988, pp. 311-320, reports chimeric genes containing the mMtI promoter linked to either rGH or hGH genes or the calcitonin/CGRP gene are expressed in very similar  
5 patterns of neuronal regions. It is suggested that the ectopic expression which is unexpected is due to regulatory signals from multiple DNA elements; that is, the interplay between the mMtI promoter and the 3' region of growth hormone gene bring about expression.

Evans, R.M. et al., "Inducible and Developmental Control of Neuroendocrine Genes", *Cold Springs Harbor Symp. Quant. Biol.*, Vol. 50, pp. 389-397, report that the localized pattern  
10 of expression of fusion genes containing the mMtI promoter and 3' untranslated flanking regions of growth hormone genes results from the combination of such gene elements in fusion genes.

Kang, J., et al., "The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor", *Nature*, Vol. 325, 19 February 1987, pp. 733-736, report the isolation and sequence of a full length cDNA clone encoding a 695-residue precursor of the amyloid proteins  
15 subunit A4.

Ponte, P., et al., "A new A4 amyloid mRNA contains a domain homologous to serine proteinase inhibitors", *Nature*, Vol. 331, 11 February 1988, pp. 525-527, disclose a novel gene encoding AD protein. The novel precursor is longer than the AAP<sub>695</sub>. It contains an additional 168 base-pair insert, encoding a 56 amino acid domain within the so-called extracellular region of  
20 the protein.

Kitaguchi, N., et al., "Novel precursor of Alzheimer's disease amyloid protein shows protease inhibitory activity", *Nature*, Vol. 331, 11 February 1988, pp. 530-532, report a novel precursor of the amyloid protein A4. This novel precursor is longer than AAP<sub>751</sub>. It contains an additional 57 base pairs encoding a 19 amino acid domain of unknown function, inserted  
25 immediately C-terminal to the insert in AAP<sub>751</sub>.

Selkoe, D. J., "Deciphering Alzheimer's Disease: The Amyloid Precursor Protein Yields New Clues", *Science*, Vol. 248, pp. 1058-1060, provides a review of AAP genes and proteins. It is reported that the gene occurs in three forms, AAP<sub>695</sub>, AAP<sub>751</sub>, and AAP<sub>770</sub> and a discussion of the conversion from precursor to the amyloid  $\beta$ -protein is included.

30 Wurtman, R.J., "Alzheimer's Disease". *Scientific American* 252:62-74, provides a review of six hypotheses which underlie the current focus on research on AD. The abnormal protein model that is reported in the reference discusses the presence of amyloid deposits in the brains of patients afflicted with Alzheimer's disease.

Glennner, G.G., "The Pathobiology of Alzheimer's Disease", *Ann. Rev. Med.* 40:45-51  
35 (1989), provides a review of the pathology of AD. The role of the  $\beta$ -protein as the major component of amyloid fibrils of plaques and cerebral vessels and the paired helical filaments of

neurofibrillary tangles is discussed.

- Muller-Hill, B. et al, "Molecular Biology of Alzheimer's Disease", Annu. Rev. Biochem., 58:287-307 (1989), provide a review of the molecular biology of Alzheimer's disease. A discussion of the genes encoding the  $\beta$ -protein, referred to as A $\beta$  amyloid, is included.
- 5 Additionally, the cDNAs of AAP protein, the genes encoding AAP protein, and the link of the AAP protein with AD are discussed.

- Price, D.L. et al., "Cellular and Molecular Biology of Alzheimer's Disease", BioEssays, Vol. 10, Nos. 2 & 3, February-March 1989, pp. 69-74, provide a review of the cellular and molecular biology of Alzheimer's disease. Included is a discussion of the animal models presently
- 10 being used. The section entitled "Animal Models" reports the use of nonhuman primates, specifically aged Rhesus monkeys. The usefulness and shortcomings of these models are reported. In addition, the use of transgenic mice as a potential animal model for AD is suggested. The advantages of such transgenic mice models are outlined and research strategies using these mice are proposed. However, at page 72, column 3, line 39, it is noted that a crucial problem exists
- 15 in designing a transgene which provides tissue specific expression. The present invention overcomes this obstacle.

U.S. Patent Number 4,736,866 issued April 12, 1988 to Leder et al discloses a transgenic non-human animal having a transgene comprising an activated oncogene sequence which increases the probability of development of neoplasms in the animal.

- 20 Strojek R.M., et al, The Use of Transgenic Animal Techniques for Livestock Improvement, Genetic Engineering: Principles and Methods, J.K. Setlow, Ed. Vol. 10 New York (1988) reviews work in the area of transgenic mice. Methods are disclosed and various transgenic lines are described and discussed.

- Skangos and Bieberich, Gene transfer into mice, Advances in Genetics, 24:285-322 (1987),
- 25 provide a review of work in the area of transgenic mice. A list of reported transgenic mice species is included, listing various transgene constructs introduced into mice.

- Palmiter, R.D. et al., Nature (London) 300:611-615 (1982) refers to a transgenic mouse containing a recombinant gene comprising mMtl promoter and rGH sequences. The mMtl promoter is inducible by the presence of heavy metal. Thus, expression of the rat growth hormone
- 30 may be controlled.

#### SUMMARY OF THE INVENTION

- The present invention provides a transgenic rodent having a transgene comprising a mouse metallothionein I (mMtl) promoter operably linked to a nucleotide sequence encoding Alzheimer amyloid precursor protein (AAP protein) operably linked to a mammalian growth (GH) hormone
- 35 3'-untranslated region. The present invention also provides a recombinant DNA molecule comprising a mammalian Mtl promoter operably linked to a nucleotide sequence encoding AAP

protein operably linked to a mammalian GH 3'-untranslated region.

#### DETAILED DESCRIPTION

The major impediment to etiological studies of Alzheimer's Disease (AD) and related drug development is the lack of any suitable animal model. With the increasing evidence in the literature that amyloid deposition is an early, if not primary event in AD pathogenesis, the present invention relates to transgenic animals which will develop Alzheimer-type amyloid deposits in brain regions corresponding to those effected in AD. These animals can be used as a basis for studies of AD etiology and as a screening system for novel compounds designed to interfere with the process of amyloid deposition.

To develop an animal model according to the present invention, transgenic animals are produced which carry a transgene whose expression results in tissue-specific amyloid deposition. Expression of the transgene must occur at a high level and in specific regions of the brain in order for the transgenic animal to provide a suitable AD model.

As used herein, the term "ectopic expression" means expression of a transgene in neurons within regions of the brain which do not correspond to regions normally directed by the control sequences, i.e. the promoter and the 3'-untranslated sequence.

As used herein, the term "ectopic regulatory sequences" means those genetic regulatory sequences which when operably linked to a gene, facilitate the ectopic expression of the gene.

Transgenes according to the present invention referred to herein as "AAP transgenes" are constructed to contain ectopic regulatory sequences operably linked to an Alzheimer's Amyloid Precursor gene (AAP gene).

As used herein, the term "Alzheimer's Amyloid Precursor gene" or "AAP gene" means a nucleotide sequence which encodes an Alzheimer's Amyloid Precursor protein (AAP protein), a protein that can be processed into Amyloid  $\beta$ -protein, or the amyloid  $\beta$ -protein itself. AAP genes include genomic clones, cDNAs, synthetically produced nucleotide sequences and combinations thereof. Conventions used to represent plasmids and fragments in Charts 7-13, though unique to this application, are meant to be synonymous with conventional representations of plasmids and their fragments. Unlike the conventional circular figures, the single line figures on the charts represent both circular and linear double-stranded DNA with initiation or transcription occurring from left to right (5' to 3'). Asterisks (\*) represent the bridging of nucleotides to complete the circular form of the plasmids. Endonuclease restriction sites are indicated above the line. Gene markers are indicated below the line.

Transgenic animals carrying AAP transgenes may be produced using techniques well known by those having ordinary skill in the art. Transgenic animals carrying AAP transgenes will be genetically programmed to overexpress AAP genes in neurons of regions of the brain corresponding to those regions in humans which are effected in AD, in order to facilitate the



development of amyloid deposits. Accordingly, such transgenic animals are useful in studying AD and in drug discovery efforts. As a screening tool, the transgenic animals according to the present invention can be used to identify compounds which are useful to prevent, impede or reverse the progression of AD and the accompanying brain function loss and dementia brought upon by amyloid deposition.

The ectopic regulatory sequences are modeled after the chimeric promoter system originally described by Swanson et al. Swanson placed the structural genes for both rat and human growth hormone (GH) under the control of the mouse metallothionein-I (mMtl) promoter in transgenic mice. For unknown reasons, ectopic expression of GH occurred in the brains of these mice; specifically, in neurons within regions later discovered to correspond to many of those primarily effected in AD. The observation was made in constructions containing rat growth hormones (rGH) sequences and in constructions containing human growth hormone sequences (hGH). This was an unexpected observation since neither mMtl nor GH are normally expressed in these neurons. It was subsequently found that when GH coding sequences were replaced by those for calcitonin/CGRP (calcitonin gene-related peptide), a similar pattern of ectopic expression was obtained as long as the mMtl promoter and the GH 3'-untranslated region (3'-UTR) flanked the cDNA. It therefore appears that some undefined interaction between these sequences directs the expression of inserted cDNAs in the neurons of brain regions of the brain that degenerate in AD.

The GH 3'-UTR from several species has been shown to provide similar results. Accordingly, transgenes according to the present invention may comprise any mammalian GH 3'-UTR sequences.

Essential to the present invention is the ectopic expression of the gene introduced in the transgenic animal. This ectopic expression is accomplished by the unexpected interaction of the promoter and the 3'-UTR of the ectopic regulatory sequences.

A transgenic animal according to the present invention will have the predisposition to develop Alzheimer's-related brain amyloidosis. Thus, an essential feature of the present invention is a transgene which contains a gene that encodes a protein or preprotein which, when expressed ectopically, results in the brain amyloidosis condition.

The amyloid protein, also referred to as the  $\beta$ -protein, is a 42-43 amino acid protein that is originally expressed as a precursor protein. Three different forms of precursor proteins have been identified. The dominant form in brain tissue is produced by translation of mRNA encoding a 695 amino acid polypeptide. Two other forms have also been described: one contains 751 amino acids, the other contains 770. The present invention uses any of the three precursor forms in the transgene. When expressed, each precursor form is subsequently processed to generate the amyloid deposit.

Each of the three precursors used contain a transmembrane domain. When the native AAP

protein is produced, it is thought to be partially secreted out of the cell. Three contiguous lysine residues, c-terminal to the single domain effectively serves as a cytoplasmic anchor, preventing full secretion of the molecule. In addition to transgenes made using the native AAP coding sequences, transgenes were also made using modified AAP coding sequences. Each of the three AAP coding  
5 nucleotide sequences were subjected to mutagenesis to convert a codon in the transmembrane domain into a stop codon. The modified AAP coding sequences when expressed produce truncated proteins that no longer contain the cytoplasmic anchor. These truncated AAP proteins are secreted.

The starting materials used to produce transgenes and transgenic animals according to the present invention are readily available to one having ordinary skill in the art. Metallothionein-I  
10 promoters are well known in the art. The mMtl promoter is well known in the art and can be purchased (Nichols Institute) or readily obtained from natural sources by those having ordinary skill in the art using well known techniques. Similarly, mammalian GH gene 3'-untranslated region sequences are readily available. Such sequences are well known and can be purchased (Nichols  
15 Institute) or readily obtained from natural sources by those having ordinary skill in the art using well known techniques. Any of the three forms of the AAP gene are also readily obtained from natural sources by those having ordinary skill in the art using well known techniques. Chart 1 shows the amino acid sequence of AAP<sub>695</sub>. Chart 2 shows the cDNA nucleotide sequence encoding AAP<sub>695</sub>. Chart 3 shows the amino acid sequence of AAP<sub>751</sub>. Chart 4 shows the cDNA  
20 nucleotide sequence encoding AAP<sub>751</sub>. Chart 5 shows the amino acid sequence of AAP<sub>770</sub>. Chart 6 shows the cDNA nucleotide sequence encoding AAP<sub>770</sub>. This sequence can be used by one having ordinary skill in the art to obtain a copy of the gene. Alternatively, one having ordinary skill in the art can produce a transgene according to the present invention or one or more components of the transgene by synthesizing the nucleotide sequences using well known nucleotide sequence synthesizer technology.

25 Transgenic animals are animals which have integrated foreign DNA in their somatic cells and germ cells. The most common way of introducing the foreign DNA into the animal is by either microinjection or retroviral infection of the animal when it is in an embryonic state. The foreign DNA then integrates itself into the genetic material of the animal after which it is replicated along with the native genetic material of the animal during the development and life of the animal.  
30 Additionally, because the foreign DNA is integrated into the germ cell DNA, the offspring of such an animal will contain copies of the foreign DNA. Transgenic animals according to the present invention can be made following the procedure described in U.S. Patent No. 4,873,191 issued October 10, 1989 to Wagner et al., which is incorporated herein by reference.

The present invention provides an AAP operably linked to ectopic regulatory sequences.  
35 Constructs according to the present invention contain mammalian Mtl promoters operably linked to AAP genes operably linked to mammalian GH3'-UTR sequences. Optionally, nucleotide

sequences enclosed mammalian GH signal sequences, including the intron contained therein, operably linked upstream of the AAP gene are included in the present invention. Rodent species, especially rats, are particularly useful, since rats provide a wider array of behavioral and physiological paradigms than mice. Contemplated equivalents include transgenes that contain  
 5 ectopic regulatory sequences operably linked to incomplete fragments of the AAP gene such that expression of the transgene results in formation of amyloidosis conditions. Contemplated equivalents of animal models according to the present invention include other non-human mammals which comprise the ectopic AAP transgene and equivalents thereof.

Example 1 Production of transgenic mice with transgene pNAN

10 Construction of pNAN

The first transgene construct described herein is referred to as pNAN. The transgene contains coding sequences from AAP<sub>695</sub> operably linked to and between the mMtl promoter linked to the sequence encoding the bovine growth hormone signal sequence, including the intron contained therein, and the 3' flanking regions of the bovine growth hormone (bGH) gene. The  
 15 transgene was constructed by inserting a fragment of AAP<sub>695</sub> consisting bases 1923-2233 into a plasmid, pBGH-10, which contains the appropriate ectopic regulatory sequences.

The vector pBGH-10 is described in Kelder, B. et al. Gene 76:75-80 (1989) which is incorporated herein by reference. pBGH-10 contains the bGH structural gene placed under the control of the mMtl promoter.

20 AAP sequences used were subclones of AAP<sub>695</sub> cDNA obtained from a human brain cDNA library. The human brain cDNA library, and appropriate host cells were from Clontech (#H11003, lot #2002). Clone Lambda SADE-1 was obtained from human brain cDNA library by hybridization with oligonucleotides BDG-1, BDG-2, BDG-4 and BDG-5.

BDG-1 5'-ccaattttgatgatgaacttcatactctgagtcgcatgctg-3'

25 BDG-2 5'-gttctgcatctgtcaaagaactgtaggttgatttcg-3'

BDG-4 5'-ctcggtcggcagcagggcgggcatcaacaggctcaacttc-3'

BDG-5 5'-cagagatctcctcgttggatattgtcaaccagaacc-3'

A subclone, pSADE-1B, was constructed by inserting into pUC13 an EcoRI fragment from Lambda SADE-1 approximately 780 bp's extending from AAP<sub>695</sub> bases 1941 to about 2700.

30 Plasmids described as shown in Chart 7.

Subclone AAP sequences from pSADE-1B were inserted within the bGH gene of pBGH-10. The coding sequence for the entire bGH signal sequence was maintained, including the intron contained within this region of the gene. The AAP sequences replaced codons #1-188 of mature bGH, maintaining the last three bGH codons plus its termination codon and 3'-UTR.

35 The inserted AAP sequence corresponded to AAP<sub>695</sub> bases 1923-2233. This was accomplished by appropriately adapting an EcoRI-MaeI fragment (bases 1941-2233) from Lambda pSADE-1B,

-10-

replacing the bGH segment in pBGH-10 from the NarI site at position 648 to the PvuII site at position 1942. The remaining AAP bases 1923-1940 were provided by the adaptors.

To form the 5'-insertion site, the bGH gene in pBGH-10 was cleaved with restriction enzyme NarI. The AAP gene fragment from pSADE-1B was cut with MaeI, flush ended, and  
5 cleaved with restriction enzyme EcoRI. The 3' end of the pBGH-10 NarI fragment was linked to the 5' end of the pSADE-1B EcoRI fragment by inserting previously annealed oligos BDG-41 and BDG-42.

BDG-41      5'-cgaagtgaagatggatgcag-3'

BDG-42      5'-aattctgcattcattcactt-3'

10 The ligation of the fragments and the oligos resulted in the 5' insertion of AAP fragment into bGH. To form the 3'-insertion site the bGH gene was cleaved with the restriction enzyme PvuII. The 5' pBGH-10 PvuII fragment was ligated to the 3' flush ended MaeI fragment of pSADE-1B to form the 3' insertion of AAP into bGH.

The methods performed to generate completed construct are well known. The order of  
15 steps followed can be summarized as:

AAP:

1. Cut pSADE-1B DNA with MaeI.
2. Flush-end pSADE-1B MaeI fragments.
3. Cut fragments from step 2 with EcoRI.

20 Adaptors:

4. Phosphorylate BDG-42 with T4 polynucleotide kinase.
5. Anneal phosphorylated BDG-42 with BDG-41.

AAP + adaptors:

6. Ligate annealed oligos to DNA fragments from step 3.
- 25 7. Gel-purify appropriate 292 base-pair fragment.

pBGH-10:

8. Cut with PvuII + NarI.
9. Gel-purify appropriate fragment.

Final construction:

- 30 10. Ligate fragments from steps 7 and 9.
11. Transform into E. coli.
12. Sequence junctions by the standard techniques. The bGH-AAP 5'-junction was sequenced from the EcoRI site at AAP position 1941. The AAP-bGH 3'-junction was sequenced from the Asp718 site in the bGH 3'-UT.

35 The usefulness of pNAN construct was determined by *in vitro* transcription/translation of the pNAN sequence. For this purpose, the intron interrupting the bGH signal sequence had to be

removed, and the sequence to be expressed was placed within a vector, pSP72 (Promega), that is suitable for transcriptional analyses.

The plasmid pNAN was cut and ligated to annealed oligos BDG-78 and BDG-79.

BDG-78 5'-agcttaccagctatgatggctgcaggcccccg-3'

5 BDG-79 5'-gtccggggcctgcagccatcatagctgta-3'

To summarize the construction for transcription/translation experiments:

1. Isolate the 956 bp pNAN/AvaII-ClaI fragment.
2. Anneal oligos BDG-78 and BDG-79 create a HindIII site at their 5' end.

In a 3-way reaction, ligate these annealed oligos, the 956 bp fragment and pSP72 cut with  
10 HindIII and ClaI.

3. Transform into *E. coli* and confirm by sequence analysis using the universal SP6 primer. This clone was called pSPNAN2.

Translation of capped *in vitro*-generated transcripts in the presence of <sup>35</sup>S-Met yielded radiolabeled protein which migrated at approximately 18-19 Kd on SDS-polyacrylamide gels.

15 When translated in the presence of microsomal membranes, the band shifted slightly but perceptibly toward a lower molecular weight. This indicates appropriate initiation and cleavage of the signal sequence in the presence of the microsomal membranes.

To generate transgenic animals, the transgene segment was generated as follows. Asp718 sites exist within the mMtI promoter and the bGH 3'-UTR. The entire transgene was liberated  
20 from pNAN as an Asp718 fragment containing approximately 700 bp mMtI promoter, the 5'-flanking sequence of the bGH gene, the bGH-AAP segments described above, plus approximately 260 base pairs bGH 3'-UTR. This fragment is introduced into mouse embryos using the methods described in Wagner, T.E. et al, Microinjection of a rabbit  $\beta$ -globin gene into zygotes and its subsequent expression in adult mice and their offspring. Proc. Natl. Acad. Sci. USA Vol. 78, No.  
25 10 pp.6376-6380, (Oct. 1981), and U.S. Patent Number 4,873,191 issued Oct. 10, 1989 to Wagner, both incorporated herein by reference.

Two hundred thirty oocytes were microinjected with the construct and transferred into nine pseudo-pregnant female recipients. DNA was collected from the tails of 60 offspring and four were shown to contain the transgene (three males, one female). This was determined by Southern  
30 blot analysis of 10  $\mu$ g DNA samples cut with Asp718, probed with radiolabeled pSADE-1B insert. These four F<sub>0</sub> mice were bred with non-transgenic cohorts and a similar analysis was done on tail DNA obtained from the resulting F<sub>1</sub> offspring to determine whether the transgene was transmitted through the germ line. Two of these founders did transmit the transgene and the resulting lines were bred to homozygosity for the transgene array. Selected homozygous and heterozygous mice  
35 were placed on 76 mM ZnSO<sub>4</sub>, while others were maintained on water without zinc. Following anesthetization by inhalation with metofane, brains were removed and RNA extracted.

Densitometric analysis of Northern blots probed with the pSADE-1B insert revealed that homozygotes expressed 2-3 fold more transgene-coded RNA than heterozygotes. Moreover, zinc intake resulted in a 1-3 fold increase in transgene-coded RNA relative to littermates containing the same transgene copy number which were maintained without zinc.

## 5 Example 2 AAP cDNAs and Modifications

Each of the three known AAP cDNAs were used as starting materials for constructions of transgenes. In addition to using subclones of each form of precursor, modifications were made to each form to insert a stop codon in the AAP coding sequences upstream from the region of the gene which encodes the cytoplasmic carboxyl terminus. When expressed, these modified AAP subclones produce molecules lacking the cytoplasmic anchor normally found in the AAP protein. Thus, the modified genes will produce modified proteins that are secreted.

For each AAP gene, cDNA was first isolated from a human brain cDNA lambda phage library using oligonucleotides BDG-1, BDG-2, BDG-4 and BDG-5. The AAP-encoding cDNA was then subcloned into pUC13 plasmids to facilitate further manipulations.

## 15 AAP<sub>695</sub> Plasmids:

To generate a full length clone of AAP<sub>695</sub>, the N-terminal portion was recovered by amplifying cDNA from Alzheimer brain RNA using PCR. The C-terminal portion was recovered by subcloning a cDNA obtained from a human brain cDNA library.

Lambda SADE-1 which contains the AAP<sub>695</sub> cDNA was obtained from human brain cDNA library by hybridization with oligonucleotides BDG-1, BDG-2, BDG-4 and BDG-5. Lambda SADE-1 extend from AAP<sub>695</sub> bases 996 to approximately 2700. The 3'-terminus was not accurately established but this was unnecessary for further work.

Two subclones were obtained from Lambda SADE-1: pSADE-1A and pSADE-1B. In both cases, the AAP<sub>695</sub> sequence from Lambda SADE-1 was subcloned into the EcoRI site of pUC13. Lambda SADE-1 was cut with EcoRI and the 947 bp EcoRI fragment extending from AAP<sub>695</sub> bases 996-1942 was inserted into pUC13, generating plasmid pSADE-1A. Plasmid pSADE-1B is the subclone of Lambda SADE-1 EcoRI fragment into pUC13 which contains approximately 780 bp EcoRI fragment extending from AAP<sub>695</sub> bases 1943 to about 2700.

Plasmid pSADE-3 which contains AAP<sub>695</sub> bases 131-1243 was derived from single stranded cDNA that was generated from Alzheimer brain RNA using BDG-75 as a primer, and double-stranded cDNA generated by PCR using BDG-74 and BDG-75 on cDNA template. The AAP sequences were subcloned as an EcoRI fragment into pUC13.

BDG-74 5'-gggaattccccgcgcagggtcgcg-3'

BDG-75 5'-gggaattcgattccactttctcctg-3'

35 Plasmid pSADE-4 contains AAP<sub>695</sub> bases 131-1942. The subcloned EcoRI inserts from pSADE-3 and pSADE-1A were inserted into pBR322 cut with EcoRI in 3-way ligation to generate

the insert that includes AAP<sub>695</sub> bases 131-1942.

Plasmid pSADE-695 was constructed next. pSADE-695 contains AAP<sub>695</sub> bases 131 to about 2700 which constitutes essentially the full length coding sequence. To construct pSADE-695, EcoRI inserts from pSADE4 and pSADE1B were subcloned into pBR322 cut with EcoRI. This was performed as a 3-way ligation.

Plasmid pSP695F contains the same AAP<sub>695</sub> insert as pSADE-695 subcloned into pSP73. Sense strand orientation reads 5' to 3' from the SP6 promoter. Plasmid pSP695R contains the same AAP<sub>695</sub> insert as pSP695F except reverse orientation, i.e. sense strand orientation reads 5' to 3' from the T7 promoter.

Plasmid pSP695R-TL-f was derived from pSP695R. In order to remove the ATG codon between the T7 promoter and the AAP initiation codon, the plasmid was cut with SalI and HindIII, flush-ended and religated. The HindIII site was regenerated and the ATG codon was deleted.

Plasmid pSP<sub>695</sub>R-TL-s was engineered to encode a secreted form of AAP<sub>695</sub> by replacing the valine codon that is two positions downstream of the amyloidogenic domain (AAP<sub>695</sub> amino acid 640) with a termination codon. This functionally deletes the C-terminal 56 amino acids including nine amino acids of the transmembrane domain, the cytoplasmic anchor and the entire cytoplasmic domain. To construct pSP695R-TL-s, the 565 bp EcoRI-SpeI fragment of pSADE-1B (AAP<sub>695</sub> bases 1941-2504) was subcloned into M13mp18 and mutagenized by site-directed mutagenesis using oligo BDG-80.

BDG-80 5'-catagcgacatagatcgatcacc-3'

The corresponding fragment was removed from pSP<sub>695</sub>R-TL-f by limit digestion with SpeI plus partial digestion with EcoRI, and replaced by this mutagenized fragment. In addition, sites for SphI, PstI, AccI and SalI were also deleted.

Plasmids pSP695R-TL $\Delta$ B-f and pSP695R-TI $\Delta$ B-s are clones that contain a BamHI site deletion at AAP<sub>695</sub> position 1475. These were generated for use in the pSAR constructions. The site is deleted without altering the coding sequence. A 1600 bp SacI fragment was subcloned from pSP695R-TL-f into M13mp19 (fragment extends from SacI site in vector polylinker through AAP<sub>695</sub> bases 131-1738), then mutagenized by site-directed mutagenesis with oligo DEL-2.

DEL-2 5'-gcatgggtggacccaagaaa-3'

The AccI-SacI fragment (AAP<sub>695</sub> bases 73-1738) in pSP695R-TL-f and pSP695R-TL-s were replaced with the corresponding mutagenized fragment.

Plasmid pAAP-695 $\Delta$ B-f was constructed by subcloning the NruI-SpeI fragment of pSP695R-TL $\Delta$ B-f (AAP<sub>695</sub> bases 144-2504) into pGEM-5Zf(+)/EcoRV-SpeI.

Plasmid pAAP-695 $\Delta$ B-s was constructed by replacing the EcoRI-SpeI fragment of pAAP-695 $\Delta$ B-f (AAP<sub>695</sub> bases 1941-2504) with EcoRI-SpeI fragment of pSP695-RTL $\Delta$ B-s. This was then moved into the vector pGEM-5Zf(+) cleaved with EcoRV and SpeI as an NruI-SpeI fragment,

deleting the EcoRV and NruI sites, but maintaining the SpeI site and placing the entire construct immediately downstream of an NcoI site necessary for further steps in the construction.

#### AAP<sub>751</sub> Plasmids

Plasmid pSADE-5 which contains AAP<sub>751</sub> bases 131-1411 was derived from single stranded cDNA that was generated from Alzheimer brain RNA using BDG-75 as a primer, and double-stranded cDNA generated by PCR using BDG-74 and BDG-75 on cDNA template.

BDG-74      5'-gggaattccccgcgcagggtcgcg-3'

BDG-75      5'-gggaattcgattccactttctcctg-3'

The AAP sequences were subcloned as an EcoRI fragment into pUC13.

Plasmid pSADE-7 contains AAP<sub>751</sub> bases 131-2110. The subcloned EcoRI inserts from pSADE-5 and pSADE-1A were inserted into pBR322 cut with EcoRI in 3-way ligation to generate the insert that includes AAP<sub>751</sub> bases 131-2110.

Plasmid pSP751R-TL-f was constructed to replace a portion of AAP<sub>695</sub> in pSP695R-TL with a corresponding portion from AAP<sub>751</sub>. The AccI-XhoI fragment of pSP695R-TL (AAP<sub>695</sub> bases 373-1056) was removed by limit digestion with AccI plus partial digestion with XhoI due to the presence of another XhoI site in the vector polylinker. This fragment was replaced by the AccI-XhoI fragment of clone pSADE-5 (AAP<sub>751</sub> bases 373-1224) to generate pSP751R-TL.

Plasmid pSP751R-TL-s was engineered to encode a secreted form of AAP<sub>751</sub> by replacing the valine codon that is two positions downstream of the amyloidogenic domain (AAP<sub>695</sub> amino acid 640) with a termination codon. This functionally deletes the C-terminal 56 amino acids including nine amino acids of the transmembrane domain, the cytoplasmic anchor and the entire cytoplasmic domain. To construct pSP751R-TL-s the 565 bp EcoRI-SpeI fragment of pSADE-1B (AAP<sub>695</sub> bases 1941-2504) was subcloned into M13mp18 and mutagenized by site-directed mutagenesis using oligo BDG-80.

BDG-80      catagcgacatagatcgtcatcacc

The corresponding fragment was removed from pSP751R-TL by limit digestion with SpeI plus partial digestion with EcoRI, and replaced by this mutagenized fragment.

Plasmid pAAP-751ΔB-f was constructed by replacing the Asp718-XhoI fragment of pAAP-695ΔB-f (AAP<sub>695</sub> bases 203-1056) with Asp718-XhoI fragment of pSP751R-TL-f (AAP<sub>751</sub> bases 203-1225).

Plasmid pAAP-751ΔB-s was constructed by replacing the Asp718-XhoI fragment of pAAP-695ΔB-s (AAP<sub>695</sub> bases 203-1056) with Asp718-XhoI fragment of pSP751R-TL-f (AAP<sub>751</sub> bases 203-1225).

#### AAP<sub>770</sub> Plasmids

Plasmid pSADE-6 which contains AAP<sub>770</sub> bases 131-1468 was derived from single stranded cDNA that was generated from Alzheimer brain RNA using BDG-75 as a primer, and



double-stranded cDNA generated by PCR using BDG-74 and BDG-75 on cDNA template. The AAP sequences were subcloned as an EcoRI fragment into pUC13.

BDG-74      5'-gggaattccccgcgcagggtcgcg-3'

BDG-75      5'-gggaattcgattccactttctctg-3'

- 5      Plasmid pSADE-8 contains AAP<sub>770</sub> bases 131-2167. The subcloned EcoRI inserts from pSADE-6 and pSADE-1A were inserted into pBR322 cut with EcoRI in 3-way ligation to generate the insert that includes AAP<sub>770</sub> bases 131-2167.

- Plasmid pSP770R-TL-f was constructed to replace a portion of AAP<sub>695</sub> in pSP695R-TL with a corresponding portion from AAP<sub>770</sub>. The AccI-XhoI fragment of pSP695R-TL (AAP<sub>695</sub> bases 373-1056) was removed by limit digestion with AccI plus partial digestion with XhoI due to the presence of another XhoI site in the vector polylinker. This fragment was replaced by the AccI-XhoI fragment of clone pSADE-6 (AAP<sub>770</sub> bases 373-1224) to generate pSP770R-TL.

- Plasmid pSP770R-TL-s was engineered to encode a secreted form of AAP<sub>770</sub> by replacing the valine codon that is two positions downstream of the amyloidogenic domain (AAP<sub>695</sub> amino acid 640) with a termination codon. This functionally deletes the C-terminal 56 amino acids including nine amino acids of the transmembrane domain, the cytoplasmic anchor and the entire cytoplasmic domain. To construct pSP770R-TL-s, the 565 bp EcoRI-SpeI fragment of pSADE-1B (AAP<sub>695</sub> bases 1941-2504) was subcloned into M13mp18 and mutagenized by site-directed mutagenesis using oligo BDG-80.

- 20      BDG-80      5'-catagcgacatagtcgtcatcacc-3'

The corresponding fragment was removed from pSP770R-TL by limit digestion with SpeI plus partial digestion with EcoRI, and replaced by this mutagenized fragment.

- Plasmids pAAP-770ΔB-f were constructed by replacing the Asp718-XhoI fragment of pAAP-695 ΔB-f (AAP<sub>695</sub> bases 203-1056) with Asp718-XhoI fragment of pSP770R-TL-f (AAP<sub>770</sub> bases 203-1281).

Plasmids pAAP-770ΔB-s were constructed by replacing the Asp718-XhoI fragment of pAAP-695ΔB-s (AAP<sub>695</sub> bases 203-1056) with Asp718-XhoI fragment of pSP751R-TL-f (AAP<sub>770</sub> bases 203-1281).

### Example 3      pSAR

- 30      Several transgenes were constructed containing nucleotide sequences from rat growth hormone (rGH). A vector, pSAR, was constructed which contains the mMTI promoter, the rGH signal sequence including the intron contained therein, and the rGH3'-UTR. Plasmid pSAR contains cloning sites which allow for insertion of AAP coding sequences which can then be expressed when the transgene constructed is liberated and used to generate a transgenic animal.

- 35      In order to construct a transgene according to the present invention using the rGH 3'-untranslated sequences, the growth hormone sequences must be modified. Thus, 5 segments of the

-16-

rGH gene were subcloned into five different plasmids to facilitate manipulations. The five subclones were modified and ligated back together to produce a modified rGH sequence. A mouse mMtI promoter was then inserted upstream of the rGH material. The mMtI promoter was recovered from starting material and amplified using PCR technology which allowed for the generation of a SmaI site at the 3' end which is not naturally present. This SmaI site was useful in the ligation of the mMtI promoter to the rGH sequence. To complete the transgene construction, an internal portion of the rGH sequence was deleted and one of the six versions of the AAP sequence was inserted in its place. The inserted AAP sequence was then modified to place it in proper reading frame for expression in transgenic animals. Charts 8-12 illustrate plasmids constructed to make pSAR.

The starting material for the rGH 3'-UT was a rat growth hormone structural gene clone in bacteriophage Lambda-Charon 4A described in Chien, Y.-H. & E.B. Thompson, Proc. Natl. Acad. Sci. USA 77:4583-4587 (1980). Aliquots of this DNA were packaged using standard techniques, amplified, and DNA was extracted from the resulting bacteriophage preparations. The DNA was digested with both BamHI and XhoI. Fragments which migrated on agarose gels at about 5 Kb were purified. These fragments were subcloned into pSP73 (Promega) cut with the BamHI and XhoI (see Chart 8). Appropriate clones, designated pRGH, were identified by hybridization with BDG-86.

BDG-86 5'-caagaggctggtgctttccctgccatgcc-3'

The pRGH clone was divided into five fragments of workable size and complexity to enable appropriate modifications. Numbering was according to the rGH sequence coordinates:

- #1 XhoI-PvuII fragment (407-789)
- #2 PvuII-PstI fragment (789-1714)
- #3 PstI-PstI fragment (1714-2564)
- #4 PstI-PstI fragment (2564-3764)
- #5 PstI-BamHI fragment (3764-5644)

Modifications were performed on PstI-PstI fragment #3 to enable insertion of the 3'-AAP sequences, including the AAP stop codon, immediately upstream of the rGH 3'-UT. These modifications were termed Step 1. The PvuII site within the 5th codon upstream of the rGH stop was selected as an insertion site. Since several PvuII sites exist within the rGH gene, it was necessary to mutate this one to enable insertion of the AAP cDNAs without further fragmentation of rGH sequences in the cloning vector. HpaI was chosen to replace PvuII. It was selected because the enzyme used needed to cut uniquely within rGH, and generate a flush-ended terminus to enable proper insertion of the AAP cDNAs. Replacement of the PvuII site with a HpaI site was accomplished using a PCR-based protocol.

New cloning vectors were generated for this section. pSP72ΔK was generated by cutting

pSP72 (Promega) with Asp718, flush-ending and recircularizing. pSP72 $\Delta$ KH was generated by cutting pSP72 $\Delta$ K with HpaI + EcoRV and recircularizing.

The Step 1 segment was initially subcloned in Bluescript (Stratagene) as a PstI fragment referred to as pStep-PPP (see Chart 8). The strategy used to replace the PvuII site at rGH 2373 employed PCR on two segments of this clone.

The 5' PstI-PvuII segment was mutated by amplifying the insert of pStep1 in pStep-PPP using oligos BDG-158 and BDG-156.

BDG-156      5'-ggggaattcgtaactgcttccgcaaagcgcg-3'

BDG-158      5'-cagccctaactgcagctaggcca-3'

BDG-158 corresponds to the rGH sequence surrounding the PstI site at position 1714. BDG-156 contains the rGH antisense sequence surrounding position 2373 (downstream of the PvuII site), but replaces the PvuII site with a HpaI site so that the amplified products contain the HpaI site in place of the PvuII site. BDG-156 also contains an EcoRI site downstream of the HpaI site to facilitate cloning. This PCR-generated fragment was subcloned into pUC13-SmaI as a blunt-ended fragment to generate pStep1-5'. It was inserted in such an orientation that there was an EcoRI site in the vector upstream of the 5'-end of this fragment, i.e. this fragment was now flanked by EcoRI sites. Insertion was random, i.e. it was in both orientations. This orientation was selected since it was the one useful for the construct. The EcoRI site at the 5' end was supplied by the vector, since it exists upstream of the SmaI site used for insertion. The EcoRI site at the 3'-end was created with the PCR primer, adjacent to the HpaI site on that primer.

The 3'-segment was mutated by amplifying the insert of pStep1-3' using oligos BDG-157 and BDG-159. BDG-159 corresponds to a cloning vector sequence 3' to the insert. BDG-157 contains the rGH sense strand sequence surrounding position 2373 (upstream of the Pvu site), but replaces the PvuII site with a HpaI site so that the amplified products contain the HpaI site in place of the PvuII site. BDG-157 also generates an EcoRI site upstream of the HpaI site to facilitate cloning. This PCR-generated fragment was cut with PstI + EcoRI and cloned into pSP72 $\Delta$ KH cut with PstI + EcoRI.

BDG-157      5'-cccgaattcgtaacgtgtgctttctaggcacacac-3'

BDG-159      5'-gacgttgtaaaacgacggccagt-3'

Oligos BDG-156 and BDG-157 were designed so that the two PCR-generated Step 1 segments could be cut with HpaI and ligated together to yield the appropriate PvuII to HpaI modification at rGH position 2373. This was accomplished by cutting pStep 1-5' and pStep 1-3' with EcoRI and HpaI. The 5' portion of fragment #3 from pStep1-5' was subcloned into the 3' portion contained in pStep1-3' to generate the final Step1 plasmid, pStep1-PHP.

Modifications on the XhoI-PvuII fragment (407-789), termed Step 2 modifications were performed to permit insertion of the 5' AAP terminus near the first rGH codon downstream of the

rGH signal peptidase cleavage site. This was accomplished by generating an NcoI site in rGH. The AAP sequences were then cloned into this site by using the immediately upstream NcoI site in vector pGEM-5Zf(+), in the pAAP series of constructs. These constructs were further modified by inserting appropriate oligonucleotide adaptors between the engineered rGH NcoI site and the  
 5 natural AAP Asp718 site, so that the AAP sequence will begin with the first codon of the mature protein, expressed as a fusion with the first 5 rGH residues. This is designed so that the rGH signal sequence should be clipped within an rGH milieu.

pStep2 is the XhoI-PvuII rGH segment (coordinates 407-789) containing the engineered NcoI site at coordinate 736, cloned as a flush-ended PCR-generated fragment into pUC13-SmaI  
 10 (see Chart 9). To construct pStep2, the 382 bp XhoI-PvuII fragment was cloned into M13 cut with XhoI and SmaI. The NcoI site was engineered by site-directed mutagenesis using oligo BDG-112.

BDG-112      5'-ccctgccatggccttgctcag-3'

The mutated insert was then excised from the M13 clones by PCR from using primers  
 15 BDG-122 & 123 to preserve XhoI site and regenerate the PvuII site.

BDG-122      5'-cagcagccagctgggtcaggtgctgggctc-3'

BDG-123      5'-tccagcaccctcgagccagattccaaact-3:

The PCR-generated segment was sequenced to ensure its integrity. The XhoI-PvuII fragment was subcloned into M13/SmaI-XhoI, and the correct sequence confirmed through the  
 20 region going into the final construct, including the presence of the engineered NcoI site.

No modifications to the nucleotide sequence were required for the PvuII-PstI fragment (789-1714) and the PstI-PstI fragment (2564-3764). However, both fragments were inserted into different vectors.

In step 3, plasmid pStep3 which contains the rGH PvuII-PstI fragment (coordinates 789-  
 25 1714) was cloned into similarly-cut pSP72 (see Chart 9).

In step 4, plasmid pStep4 which contains the rGH PstI fragment (coordinates 2564-3764) cloned into PstI-cut Bluescript M13+-SK (see Chart 9).

Modifications were performed on the PstI-BamHI fragment (3764-5644) to construct pStep5. pStep5 was produced when the rGH PstI-BamHI fragment (coordinates 3764-5644) was  
 30 cloned into similarly-cut pSP72/KH. NcoI and Asp718 sites were mapped within this rGH segment at positions 4760 and 5470, respectively. These sites must be deleted to enable appropriate cloning of AAP sequences. Since these are approximately 3000 bases away from the termination codon, it was felt that some modifications at these sites within the 3'-UTR are unlikely to be detrimental to the expressional specificity of the transgenic constructs. These sites were  
 35 individually eliminated by cutting with the enzyme, flush-ending and recircularizing (see Chart 9).

Once modification were performed on the five subclones, the fragments were relinked to

produce a modified version of the original rGH sequence. The attachment and modifications of plasmids from steps 1-5 was performed sequentially and produced a series of plasmids.

Plasmid pStep23 results from ligation of subclone XhoI-PvuII insert from pStep2 into similarly-cut pStep3, generating a plasmid containing rGH coordinates 407-1714 (see Chart 10).

- 5        Fragment 1 is then combined with pStep23 and a portion of the plasmid is deleted. Then fragments 4 and 5 are added. In order to subclone the rGH segments from steps 4 and 5 into the Step231 construct, the PstI site at position 1714 must be deleted. This is not problematic since the rGH region between coordinates 736-2373 is deleted in all pSAR-AAP constructs. However, the PstI site at position 469 within the pStep23 segment must be maintained. Therefore, the following
- 10        steps were done in order:

1. Generate two subclones which separate the PstI site at position 469 in pStep23 from the remainder of the pStep23 insert at a site unique within pRGH (BglII-PstI 1271-1714).
2. Subclone the pStep1 insert into this plasmid and do the necessary deletion.
3. Subclone the pStep45 insert into this plasmid.
- 15        4. Add back the missing pStep23 sequences.

To accomplish this plasmid pStep23-XB is constructed. PStep23-XB is a subclone of pStep23 which contains the XhoI-BglII fragment of pStep23 (rGH coordinates 407-1271) into similarly-cut pSP72 (see Chart 10).

- 20        The remaining fragment of pStep23 is pStep23-BP, the subclone containing the BglII-PstI fragment of pStep23 (rGH coordinates 1271-1714) inserted into similarly-cut pSP72 (see Chart 10).

The step 1 fragment is ligated to the pStep23-BP to form pStep23-BP-1. The step 1 fragment is the PstI fragment of pStep1-PHP (rGH coordinates 1714-2564). It is inserted into similarly-cut pStep23-BP. Orientation confirmed by analytical restriction digestions. Clone contains rGH coordinates 1271-2564 (see Chart 10).

- 25        Plasmid pStep23-BP-1 $\Delta$  is pStep23-BP-1 with the necessary deletion. In order to delete the rGH region containing PstI-1714, pStep23-BP-1 was cut with StyI and Asp718, flush-ended and recircularized. This deleted the region between rGH coordinates 1396-1907 and recreated an Asp718 site at the recircularized junction (see Chart 10).

- 30        pStep45 is the combination of fragments from steps 4 and 5. To form pStep45, the PstI fragment from pStep4 was subcloned into similarly-cut pStep5. Orientation confirmed by analytical restriction digestions. pStep45 contains rGH coordinates 2564-5644 (see Chart 11).

- 35        pStep23-BP-1 $\Delta$ -45 resulted from a 3-way ligation of: 1) the BglII-PstI insert from pStep23-BP-1 $\Delta$  (rGH coordinates 1271-2564); 2) the BamHI-PstI(partial) insert from pStep45 (rGH coordinates 2564-5604); and 3) phosphatased BamHI-BglII-cut pSP72. Orientation confirmed by analytical restriction digestions. Clone contains rGH coordinates 1271-5604 with 1396-1907 deleted (see Chart 11).

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pStep231 $\Delta$ 45 was generated by the following steps:

1. Cut pStep23-XB and pStep23-BP-1 $\Delta$ -45 with BglII.
2. Ligate these two BglII-cut plasmids.
3. Cut ligated DNAs with XhoI + BamHI and purify the 4726 bp fragment (rGH coordinates 407-5644 with 1396-1907 deleted).
4. Clone this XhoI-BamHI fragment into similarly-cut pGEM-11Zf(-), generally pStep231 $\Delta$ 45 (see Chart 11).

This completed the rGH segment of the pSAR construction. The rGH sequence could now appropriately be combined with the mMtI promoter to provide a vector in which AAP sequences can be inserted to form a working transgene.

The next step was to combine the mouse metallothionein-I promoter with the rGH segments. The starting plasmid for the metallothionein component was pXGH5 (Nichols Institute) (see Chart 12).

The desired junction between mMtI and rGH was generated by flush-ending the rGH XhoI terminus and recreating the remainder of the junction using PCR on the mMtI clone.

PCR utilizing primers BDG-68 and BDG-213 amplified an approximately 2100 bp fragment from pXGH5 containing pUC12 polylinker sequence (from pXGH5) at the 5'-end and the appropriate junction sequence at the 3'-end.

BDG-68      5'-gtttcccagtcacgac-3'  
 BDG-213     5'-gggatctggtgaagctggag-3'

The fragment was cut with EcoRI and cloned into pSP73/EcoRI-SmaI generating plasmid pSP73mMtI; the EcoRI site was supplied by the amplified polylinker sequence from pXGH5, and the SmaI site is supplied by flush-end ligation of the amplified sequence (contains half an SmaI site) to the SmaI site of pSP73. The Asp718 site must then be deleted from this mMtI segment since it will interfere with the cloning of AAP sequences into pSAR, but the change is irrelevant to the transgenic constructs since it will not be included within the transgenes (see Chart 12).

Thus, to make a construct from the plasmids described that contains the mMtI promoter sequence upstream from the 3'UTR sequence of rGH, the following plasmids were constructed.

To construct plasmid pmMtI $\Delta$ K, mMtI sequence amplified from pXGH5 with primers BDG-68 and BDG-213 was cut with EcoRI and cloned into EcoRI-SmaI-cut pSP73. The Asp718 site at mMtI position 1100 was deleted by cutting with Asp718, flush-ending and recircularizing (see Chart 12).

Plasmid pSAR was then constructed. The EcoRI-SmaI insert from pmMtI $\Delta$ K was subcloned into pStep231 $\Delta$ 45 cut with XhoI, flush-ended, then recut with EcoRI. This generated the completed pSAR cloning vector (see Chart 12). The sequence of the PCR-generated stretch of mMtI promoter was confirmed.

#### Insertion of AAP sequence into pSAR

To insert AAP fragments into pSAR, AAP clones pAAP-(695,751,770) $\Delta$ B/(f,s) were cut with MaeI. This cut the AAP clones at AAP termination codon and within pGEM5-Zf(+). MaeI fragments were flush ended, recut with NcoI, and subcloned into pSAR cut with NcoI and HpaI.

5 The following plasmids were constructed:

pSAR-695NM/f,s: AAP-695 full length and secreted constructs from pAAP-695 $\Delta$ B-s in pSAR, not yet adapted for expression.

pSAR-751NM/f,s: AAP-751 full length and secreted constructs from pAAP-751 $\Delta$ B-s in pSAR, not yet adapted for expression.

10 pSAR-770NM/f,s: AAP-770 full length and secreted constructs from pAAP-770 $\Delta$ B-s in pSAR, not yet adapted for expression. Collectively these plasmids are referred to herein as pSAR-NM.

The region between the NcoI site in the 5' rGH segment and the Asp718 site in the AAP segment had to be removed and replaced by appropriate adaptors to place the AAP sequences under  
15 proper control of the mMtI promoter and rGH signal sequence. This was accomplished as follows:

pSAR-NM was cut with NcoI and Asp718 and NcoI-Asp718 annealed adaptors BDG-173 & BDG-174 were subcloned therein.

BDG 173 5'-catgctggaa-3'

BDG 174 5'-gtacttcag-3'

20 The following plasmids were constructed:

pSAR-695/(f,s): Final AAP-695 full length and secreted constructs from pSAR-695NM/f,s, properly adapted for expression.

pSAR-751/(f,s): Final AAP-751 full length and secreted constructs from pSAR-751NM/f,s, properly adapted for expression.

25 pSAR-770/(f,s): Final AAP-770 full length and secreted constructs from pSAR-770NM/f,s, properly adapted for expression.

To generate transgenic animals, the transgene segment was generated as follows: pSAR-695/(f,s) was cut with BglI and BamHI. The entire transgene was liberated. The pSAR-695/(f,s) BglI-BamHI transgenes were microinjected fertilized mouse ali following the directions in U.S.  
30 Patent 4,873,191. pSAR-695/f was injected into 668 eggs yielding 101 pups, and pSAR-695/s into 570 eggs yielding 94 pups. Of the 101 pSAR-695/f pups, 41 were shown to be potential founders by Southern blot analysis of DNA extracted from tails. Of the 94 pSAR-695/s pups, 29 were similarly shown to potential founders.

#### Example 4 Mouse Brain Analyses

35 The brains of sacrificed transgenic mice are each analyzed as follows. Northern blot and ribonuclease protection assays on RNAs extracted from brain tissue are performed to evaluate gross

transgene expression at the RNA level. Western blot analyses using several antisera that recognize the AAP region encoded by the pNAN transgene are performed on protein extracted from brain tissue to evaluate gross transgene expression at the protein level. *In situ* hybridizations are performed on sections of brain tissue to evaluate regional and cellular-specificity transgene expression at the RNA level. Immunocytochemistry studies on sections of brain tissue are performed to evaluate regional and cellular-specificity of transgene expression at the protein level. By histological methods including but not restricted to Congo red, Thioflavin T, Thioflavin S, silver staining methods are performed to evaluate neuronal and other pathological abnormalities.



Chart 1

1 MLPG LALLLL AAWTARALEY PTDGNAGLLA EPQIAMFCGR LNMHMNVQNG  
51 KWSDPSGTK TCIDTKEGIL QYCQEYYPEL QITNVYEANQ PYTIQNWCKR  
101 GRKQCKTHPH FVIPYRCLVG EFVSDALLVP DKCKFLHQR MDVCETHLHW  
151 HTVAKETCSE KSTNLHDYGM LLPCGIDKFR GVEFVCCPLA EESDNVDSAD  
201 AEEDDSDYWW GGADTDYADG SEDKVVEVAE EEEVAEVEEE EADDDDEDDED  
251 GDEVEEEAEE PYEEATERTT SIATTTTTTT ESVEEVVRVP TTAASTPDAV  
301 DKYLETPGDE NEHAHFQKAK ERLEAKHRER MSQVMREWEE AERQAKNLPK  
351 ADKKAVIQHF QEKVESLEQE AANERQQLVE THMARVEAML NDRRRLALEN  
401 YITALQAVPP RPRHYFNMLK KYVRAEQKDR QHTLKHFEHV RMVDPKKAQ  
451 IRSQVMTHLR VIYERMNQLS SLLYNYPAYA EEIQDEYDEL LQKEQNYSD  
501 VLANMISEPR ISYGNALMP SLTETKTTVE LLPYNGEFSL DDLQPWHSFG  
551 ADSVPANTEN EVEPYDARPA ADRGLTTRPG SGLTNIKTEE ISEVKMDAEF  
601 RHDSGYEVHH QKLYFFAEDV GSNKGAIIGL MVGGVVIATV IVITLYMLKK  
651 KQYTSIHHGV VEYDAAVTP ERLSKMQQN GYENPTYKFF EQMQN•

Chart 2

1 agtttcctcg gcagcggtag gcgagagcac gcggaggagc gtgcgcgggg  
51 ccccgggaga cggcggcggt ggccggcgcg gcagagcaag gacgcggcgg  
101 atcccactcg cacagcagcg cactcggtgc cccgcgcagg gtcgcgatgc  
151 tgcccgggtt ggcaactgct ctgctggcgg cctggacggc tcgggcgctg  
201 gaggtaccca ctgatggtaa tgctggcctg ctggctgaac ccagattgc  
251 catgttctgt ggcagactga acatgcacat gaatgtccag aatgggaagt  
301 gggattcaga tccatcaggg accaaacct gcattgatac caaggaagc  
351 atcctgcagt attgccaaga agtctacct gaactgcaga tcaccaatgt  
401 ggtagaagcc aaccaaccag tgaccatcca gaactggtgc aagcggggcc  
451 gcaagcagtg caagacccat cccactttg tgattcccta ccgtgctta  
501 gttggtgagt ttgtaagtga tgcccttctc gttcctgaca agtgcaaat  
551 cttacaccag gagaggatgg atgtttgca aactcatctt cactggcaca  
601 ccgtcgcca agagacatgc agtgagaaga gtaccaactt gcatgactac  
651 ggcattgtgc tgccctgagg aattgacaag ttccgagggg tagagtttgt  
701 gtgttgcca ctggctgaag aaagtgaca tgtggattct gctgatgcg  
751 aggaggatga ctcggtatgc tggtagggcg gagcagacac agactatgca  
801 gatgggagtg aagacaaagt agtagaagta gcagaggagg aagaagtggc  
851 tgagggtgaa gaagaagaag ccgatgatga cgaggacgat gaggatggtg  
901 atgaggtaga ggaagaggct gaggaacct acgaagaagc cacagagaga  
951 accaccagca ttgccaccac caccaccacc accacagagt ctgtggaaga  
1001 ggtggttcga gttcctaca cagcagccag taccctgat gccgttgaca  
1051 agtatctcga gacacctggg gatgagaatg aacatgcccc ttccagaaa  
1101 gccaaagaga ggcttgaggc caagcaccga gagagaatgt ccaggtcat  
1151 gagagaatgg gaagaggcag aacgtcaagc aaagaacttg cctaaagctg  
1201 ataagaaggc agttatccag ctttccagg agaagtggg atctttggaa  
1251 caggaagcag ccaacgagag acagcagctg gtggagacac acatggccag  
1301 agtggagcc atgctcaatg accgcccg cctggccctg gagaactaca  
1351 tcaccgctct gcaggctgtt cctcctcggc ctgctcacgt gttcaatatg

Chart 2 (Cont'd)

1401 ctaaagaagt atgtccgcgc agaacagaag gacagacagc acaccctaaa  
1451 gcatttcgag catgtgcgca tgggtggatcc caagaagacc gctcagatcc  
1501 ggtcccaggt tatgacacac ctccgtgtga tttatgagcg catgaatcag  
1551 tctctctccc tgctctacaa cgtgcctgca gtggccgagg agattcagga  
1601 tgaagttgat gagctgcttc agaaagagca aaactattca gatgacgtct  
1651 tggccaacat gattagtga ccaaggatca gttacggaaa cgatgctctc  
1701 atgccatctt tgaccgaac gaaaaccacc gtggagctcc tccccgtga  
1751 tggagagttc agcctggacg atctccagcc gtggcattct tttggggctg  
1801 actctgtgcc agccaacaca gaaaacgaag ttgagcctgt tgatgcccg  
1851 cctgctgccc accgaggact gaccactcga ccagggtctg ggttgacaaa  
1901 tatcaagacg gaggagatct ctgaagtga gatggatgca gaattccgac  
1951 atgactcagg atatgaagtt catcatcaaa aattgggtgt ctttgacaga  
2001 gatgtgggtt caaacaaagg tgcaatcatt ggactcatgg tgggcggtgt  
2051 tgtcatagcg acagtgatcg tcatacctt ggtgatgctg aagaagaac  
2101 agtacacatc cattcatcat ggtgtggtgg aggttgacgc cgctgtcacc  
2151 ccagaggagc gccacctgtc caagatgcag cagaacggct acgaaatcc  
2201 aacctacaag ttctttgagc agatgcagaa ctgaccccc gccacagcag  
2251 cctctgaagt tggacagcaa aaccattgct tcactaccca tcggtgtcca  
2301 tttatagaat aatgtggga gaaacaacc cgttttatga tttactcatt  
2351 atcgcccttt gacagctgtg ctgtaacaca agtagatgcc tgaacttga  
2401 ttaaccaca catcagtaat gtattctatc tctctttaca ttttggctctc  
2451 tatactacat tattaatggg tttgtgtac tgtaagaat ttagctgtat  
2501 caaactagt catgaatga ttctctctg attatttatc acatagcccc  
2551 ttagccagtt gtatattatt cttgtgggtt gtgacccaat taagtcctac  
2601 tttacatatg ctttaagaat cgatggggga tgcttcattg gaacgtggga  
2651 gttcagctgc ttctcttgcc taagtattcc tttcctgac actatgcatt  
2701 ttaagttaa acatttttaa gtatttcaga tgcttttagag agattttttt  
2751 tccatgactg cattttactg tacagattgc tgcttctgct atatttgtga  
2801 tataggaatt aagaggatac acacgtttgt ttcttcgtgc ctgttttatg  
2851 tgcacacatt aggcattgag acttcaagct tttctttttt tgtccacgta

Chart 2 (Cont'd)

2901 tctttgggtc ttgataaag aaaagaatcc ctgttcattg taagcacttt  
2951 tacggggcgg gtggggaggg gtgctctgct ggtcttcaat taccaagaat  
3001 tctccaaaac aattttctgc aggatgattg tacagaatca ttgcttatga  
3051 catgatcgct ttctacactg tattacataa ataaattaaa taaaataacc  
3101 ccgggcaaga cttttctttg aaggatgact acagacatta aataatcgaa  
3151 gtaattttgg gtggggagaa gaggcagatt caattttctt taaccagtct  
3201 gaagtttcat ttatgatata aaagaagatg aaaatggaag tggcaatata  
3251 aggggatgag gaaggcatgc ctggacaaac ccttctttta agatgtgtct  
3301 tcaatttgta taaaatgggtg ttttcatgta aataaataca ttcttgaggg  
3351 agc

Chart 3

1 MLPGLALLLL AAWTARALEV PTDGNAGLLA EPQIAMFCGR LNMHMNVQNG  
51 KWSDPSGTK TCIDTKEGIL QYCQEVYPEL QITNVVEANQ PVTIQNWCKR  
101 GRKQCKTHPH FVIPYRCLVG EFVSDALLVP DKCKFLHQER MDVCETHLHW  
151 HTVAKETCSE KSTNLHDYGM LLPCGIDKFR GVEFVCCPLA EESDNVDSAD  
201 AEEDDSYVWW GGADTDYADG SEDKYVEVAE EEEVAEVEEE EADDDDEDDED  
251 GDEVEEEAEE PYEEATERTT SIATTTTTTTT ESVEEVVREV CSEQAETGPC  
301 RAMISRWYFD VTEGKCAPFF YGGCGGNRRN FDTEEYCMAY CGSAIPTTAA  
351 STPDAVDKYL ETPGDENEHA HFQKAKERLE AKHRERMSQV MREWEEAERQ  
401 AKNLPKADKK AVIQHFQEKV ESLEQEAANE RQQLVETHMA RYEAMLNDRR  
451 RLALenyITA LQAVPPRPRH VFNMLKKYVR AEQKDRQHTL KHFEHVRMVD  
501 PKKAAQIRSQ VMTHLRVIYE RMNQSLSLLY NYPAAVEEIQ DEVDELLQKE  
551 QNYSDDVLAN MISEPRISYG NDALMPSLTE TKTTVELLPV NGEFSLDDLQ  
601 PWSFGADSV PANTENEVEP YDARPAADRG LTTRPGSGLT NIKTEEISEV  
651 KMDAEFRHDS GYEVHHQKLV FFAEDVGSNK GAIIGLMVGG VVIATVIVIT  
701 LYMLKKKQYT SIHHGYVEYD AAVTPEERHL SKMQQNGYEN PTYKFFEQQM  
751 N\*

Chart 4

1 agtttccctcg gcagcggtag gcgagagcac gcggaggagc gtgcgcgggg  
51 ccccgaggaga cggcggcggt ggcggcgcg gcagagcaag gacgcggcgg  
101 atcccactcg cacagcagcg cactcggtgc cccgcgcagg gtcgcgatgc  
151 tgcccggttt ggcactgctc ctgctggccg cctggacggc tcgggcgctg  
201 gaggtacca ctgatggtaa tgctggcctg ctggctgaac ccagattgc  
251 catgttctgt ggcagactga acatgcacat gaatgtccag aatgggaagt  
301 gggattcaga tccatcagg accaaaacct gcattgatac caaggaaggc  
351 atcctgcagt attgccaaga agtctaccct gaactgcaga tcaccaatgt  
401 ggtagaagcc aaccaaccag tgaccatcca gaactgggtgc aagcggggcc  
451 gcaagcagtg caagacccat cccactttg tgattcccta ccgctgctta  
501 gttggtgagt ttgtaagtga tgcccttctc gttcctgaca agtgcaaatt  
551 cttacaccag gagaggatgg atgtttgcga aactcatctt cactggcaca  
601 ccgtcgccaa agagacatgc agtgagaaga gtaccaactt gcatgactac  
651 ggcattgtgc tgccctgcgg aattgacaag ttccgagggg tagagtttgt  
701 gtgttgccca ctggctgaag aaagtgaca tgtggattct gctgatgcgg  
751 aggaggatga ctcggtatgc tggtggggcg gagcagacac agactatgca  
801 gatgggagtg aagacaaagt agtagaagta gcagaggagg aagaagtggc  
851 tgaggtggaa gaagaagaag ccgatgatga cgaggacgat gaggatggtg  
901 atgaggtaga ggaagaggct gaggaacct acgaagaagc cacagagaga  
951 accaccagca ttgccaccac caccaccacc accacagagt ctgtggaaga  
1001 ggtggttcga gaggtgtgct ctgaacaagc cgagacgggg ccgtgccgag  
1051 caatgatctc ccgctggtac tttgatgtga ctgaaggga gtgtgccccca  
1101 ttcttttacg gcggatgtgg cggcaaccgg aacaactttg acacagaaga  
1151 gtactgcatg gccgtgtgtg gcagcgccat tcctacaaca gcagccagta  
1201 cccctgatgc cgttgacaag tatctcgaga cacctgggga tgagaatgaa  
1251 catgccatt tccagaaagc caaagagagg cttgaggcca agcaccgaga  
1301 gagaatgtcc caggatcatga gagaatggga agaggcagaa cgtcaagcaa  
1351 agaacttgcc taaagctgat aagaaggcag ttatccagca tttccaggag

Chart 4 (Cont'd)

1401 aaagtggant ctttggaa ca ggaagcagcc aacgagagac agcagctggt  
1451 ggagacacac atggccagag tggagccat gctcaatgac cgcgcgcgc  
1501 tggccctgga gaactacatc accgctctgc aggctgttcc tccctggcct  
1551 cgtcacgtgt tcaatatgct aaagaagtat gtccgcgcag aacagaagga  
1601 cagacagcac accctaagc atttcagca tgtgcgcctg gtggatccca  
1651 agaaagccgc tcagatccgg tcccagggtta tgacacacct ccgtgtgatt  
1701 tatgagcgca tgaatcagtc tctctccctg ctctacaacg tgcctgcagt  
1751 ggccgaggag attcaggatg aagttgatga gctgcttcag aaagagcaaa  
1801 actattcaga tgacgtcttg gccacatga ttagtgaacc aaggatcagt  
1851 tacggaaacg atgctctcat gccatctttg accgaacga aaaccacagt  
1901 ggagctcctt cccgtgaatg gagagttcag cctggacgat ctccagccgt  
1951 ggcattcttt tggggctgac tctgtgccag ccaacacaga aaacgaagtt  
2001 gagcctgttg atgccgcgc tctgtccgac cgaggactga ccactcgacc  
2051 aggttctggg ttgacaata tcaagacgga ggagatctct gaagtgaaga  
2101 tggatgcaga attccgacat gactcaggat atgaagttca tcatcaaaa  
2151 ttggtgttct ttgcagaaga tgtgggttca acaaaaggtg caatcattgg  
2201 actcatggtg ggcgggtgtg tcatagcgac agtgatctc atcaccttg  
2251 tgatgctgaa gaagaacag tacacatcca ttcacatgg tgtggtggag  
2301 gttgacgccc ctgtacccc agaggagcgc cacctgtcca agatgcagca  
2351 gaacggctac gaaatccaa cctacaagtt ctttgagcag atgcagaact  
2401 agacccccgc cacagcagcc tctgaagttg gacagcaaaa ccattgcttc  
2451 actaccatc ggtgtccatt tatagaataa tgtgggaaga aacaaacccg  
2501 ttttatgatt tactcattat cgccttttga cagctgtgct gtaacacaag  
2551 tagatgctg aacttgaatt aatccacaca tcagtaatgt attctatctc  
2601 tctttacatt ttggtctcta tactacatta ttaatggggt ttgtgtactg  
2651 taagaattt agctgtatca aactagtga tgaatagatt ctctcctgat  
2701 tatttatcac atagccctt agccagttgt atattattct tgtggtttgt  
2751 gacccaatta agtctactt tacatatgct ttaagaatcg atgggggatg  
2801 cttcatgtga acgtgggagt tcagctgctt ctcttgcta agtatccctt  
2851 tctgatcac tatgcattt aaagttaac atttttaagt atttcagatg

Chart 4 (Cont'd)

2901 ctttagagag attttttttc catgactgca ttttactgta cagattgctg  
2951 cttctgctat atttgtgata taggaattaa gaggatacac acgtttgttt  
3001 cttcgtgcct gttttatgtg cacacattag gcattgagac ttcaagcttt  
3051 tctttttttg tccacgtatc tttgggtcct tgataaagaa aagaatccct  
3101 gttcattgta agcactttta cggggcgggt ggggagggt gctctgctgg  
3151 tcttcaatta ccaagaattc tccaaaacaa ttttctgcag gatgattgta  
3201 cagaatcatt gcttatgaca tgatcgcttt ctacactgta ttacataaat  
3251 aaattaata aaataacccc gggcaagact tttctttgaa ggatgactac  
3301 agacattaaa taatcgaagt aattttgggt ggggagaaga ggcagattca  
3351 attttcttta accagtctga agtttcattt atgatacaaa agaagatgaa  
3401 aatggaagtg gcaatataag gggatgagga aggcattgcct ggacaaaccc  
3451 ttcttttaag atgtgtcttc aatttgtata aaatgggtgtt ttcatgtaaa  
3501 taaatacatt cttggaggag c



Chart 5

1 MLPG LALLLL AAWTARALEV PTDGNAGLLA EPQIAMFCGR LNMHMNVQNG  
51 KWSDPSGTK TCIDTKEGIL QYCQEVYPEL QITNVVEANQ PVTIQNWCKR  
101 GRKQCKTHPH FVIPYRCLYG EFVSDALLVP DKCKFLHQR MDVCETHLHW  
151 HTYAKETCSE KSTNLHDYGM LLPCGIDKFR GVEFVCCPLA EESDNVDSAD  
201 AEEDDSOVWW GGADTDYADG SEDKVVEVAE EEEVAEVEEE EADDDDEDDED  
251 GDEVEEEAEE PYEATERTT SIATTTTTTT ESVEEVVREV CSEQAETGPC  
301 RAMISRWFYD VTEGKCAPFF YGGCGGNRRN FDTEEYCMV CGSAMSQSLL  
351 KTTQEPLARD PVKLPTTAAS TPDVYDKYLE TPGDENEHAH FQKAKERLEA  
401 KHRERMSQVM REWEEAERQA KNLPKADKKA VIQHFQEKVE SLEQEAANER  
451 QQLVETHMAR VEAMLNDRRR LALenyITAL QAVPPRPRHV FNMLKKYVRA  
501 EQKDRQHTLK HFEHYRMVDP KKAQIRSQV MTHLRVIYER MNQSLSLLYN  
551 VPAYAEIIQD EYDELLQKEQ NYSDDVLANM ISEPRISYGN DALMPSLTET  
601 KTTVELLPVN GEFSLDDLQP WHSFGADSVP ANTENEVEPV DARPAADRGL  
651 TTRPGSGLTN IKTEEISEVK MDAEFRHDSG YEYHHQKLVF FAEDVGSNKG  
701 AIIGLMVGGV VIATVIVITL VMLKKKQYTS IHHGVVEVDA AVTPEERHLS  
751 KMQQNGYENP TYKFFEQMQN •

Chart 6

1 agtttctctg gcagcggtag gcgagagcac gcggaggagc gtgcgcgggg  
51 ccccgggaga cggcggcggt ggcggcgcg gcagagcaag gacgcggcgg  
101 atcccactcg cacagcagcg cactcgggtgc cccgcgcagg gtcgcgatgc  
151 tgcccggttt ggcactgctc ctgctggcgg cctggacggc tcgggcgctg  
201 gaggtaccca ctgatggtaa tgctggcctg ctggctgaac cccagattgc  
251 catgttctgt ggcagactga acatgcacat gaatgtccag aatgggaagt  
301 gggattcaga tccatcaggg accaaaacct gcattgatac caaggaaggc  
351 atcctgcagt attgccaaga agtctaccct gaactgcaga tcaccaatgt  
401 ggtagaagcc aaccaaccag tgaccatcca gaactggtgc aagcggggcc  
451 gcaagcagtg caagacccat cccactttg tgattcccta ccgctgctta  
501 gttggtgagt ttgtaagtga tgcccttctc gttcctgaca agtgcaaatt  
551 cttacaccag gagaggatgg atgtttgcga aactcatctt cactggcaca  
601 ccgtcgccaa agagacatgc agtgagaaga gtaccaactt gcattgactac  
651 ggcatgttgc tgccctgcgg aattgacaag ttccgagggg tagagtttgt  
701 gtgttgccca ctggctgaag aaagtgaaca tgtggattct gctgatgcgg  
751 aggaggatga ctcggtatgc tggtggggcg gagcagacac agactatgca  
801 gatgggagtg aagacaaagt agtagaagta gcagaggagg aagaagtggc  
851 tgaggtggaa gaagaagaag ccgatgatga cgaggacgat gaggatgggt  
901 atgaggtaga ggaagaggct gaggaacctt acgaagaagc cacagagaga  
951 accaccagca ttgccaccac caccaccacc accacagagt ctgtggaaga  
1001 ggtggttcga gaggtgtgct ctgaacaagc cgagacgggg ccgtgccgag  
1051 caatgatctc ccgtggtac tttgatgtga ctgaaggga gtgtgcccc  
1101 ttcttttacg gcggatgtgg cggcaaccgg aacaactttg acacagaaga  
1151 gtactgcatg gccgtgtgtg gcagcgccat gtcccaaagt ttactcaaga  
1201 ctaccagga acctcttgcc cgagatcctg ttaaacttcc tacaacagca  
1251 gccagtacc ctgatgccgt tgacaagtat ctcgagacac ctggggatga  
1301 gaatgaacat gccatttcc agaaagccaa agagaggctt gaggccaagc  
1351 accgagagag aatgtcccag gtcattgagag aatgggaaga ggcagaacgt

Chart 6 (Cont'd)

1401 caagcaaga acttgccata agctgataag aaggcagtta tccagcattt  
1451 ccaggagaaa gtggaatctt tggaacagga agcagccaac gagagacagc  
1501 agctgggtgga gacacacatg gccagagtgg aagccatgct caatgaccgc  
1551 cgccgcctgg ccctggagaa ctacatcacc gctctgcagg ctgttctctc  
1601 tcggcctcgt caegtgttca atatgctaaa gaagtatgtc cgcgcagAAC  
1651 agaaggacag acagcacacc ctAaagcatt tcgagcatgt gcgcatgggtg  
1701 gatcccaaga aagccgctca gatccgggtcc caggttatga cacacctcgc  
1751 tgtgatttat gagcgcatga atcagtctct ctccctgtct tacaacgtgc  
1801 ctgcagtggc cgaggagatt caggatgaag ttgatgagct gcttcagaaa  
1851 gagcaaaact attcagatga cgtcttggcc aacatgatta gtgaaccaag  
1901 gatcagttac ggaacgatg ctctcatgcc atctttgacc gaaacgaaa  
1951 ccaccgtgga gctccttccc gtgaatggag agttcagcct ggacgatctc  
2001 cagccgtggc attcttttgg ggctgactct gtgccagcca acacagaaa  
2051 cgaagttgag cctgttgatg cccgccctgc tgccgaccga ggactgacca  
2101 ctgaccagg ttctgggttg acaatatca agcggagga gatctctgaa  
2151 gtgaagatgg atgcagaatt ccgacatgac tcaggatatg aagttcatca  
2201 tcaaaattg gtgttctttg cagaagatgt ggggtcaaac aaaggtgcaa  
2251 tcattggact catgggtggc ggtgttgtca tagcgacagt gatcgtcctc  
2301 accttggtga tgctgaagaa gaaacagtac acatccattc atcatggtgt  
2351 ggtggagggtt gacgccgtg tcaccccaga ggagcgccac ctgtccaaga  
2401 tgcagcagaa cggctacgaa aatccaacct acaagttctt tgagcagatg  
2451 cagaactaga cccccgccac agcagcctct gaagttggac agcaaaacca  
2501 ttgcttcact acccatcggg gtccatttat agaataatgt ggaagaaac  
2551 aaacccgttt tatgatttac tcattatcgc cttttgacag ctgtgctgta  
2601 acacaagtag atgcctgaac ttgaattaat ccacacatca gtaatgtatt  
2651 ctatctctct ttacattttg gtctctatac tacattatta atgggttttg  
2701 tgtactgtaa agaatttagc tgtatcaaac tagtgcatga atagattctc  
2751 tcctgattat ttatcacata gcccttagc cagttgtata ttattcttgt  
2801 ggtttgtgac ccaattaagt cctactttac atatgcttta agaatcgatg  
2851 ggggatgctt catgtgaacg tgggagttca gctgcttctc ttgcctaagt

Chart 6 (Cont'd)

2901 attcctttcc tgatcactat gcatttttaa gttaaacatt tttasgtatt  
2951 tcagatgctt tagagagatt ttttttccat gactgcattt tactgtacag  
3001 attgctgctt ctgctatatt tgtgatatag gaattaagag gatacacacg  
3051 tttgtttctt cgtgcctggt ttatgtgcac acattaggca ttgagacttc  
3101 aagcttttct ttttttgtcc acgtatcttt gggctcttga taaagsaaag  
3151 aatccctggt cattgtaagc acttttacgg ggcgggtggg gaggggtgct  
3201 ctgctggtct tcaattacca agaattctcc aaaacaattt tctgcaggat  
3251 gattgtacag aatcattgct tatgacatga tcgctttcta cactgtatta  
3301 cataaataaa ttaaataaaa taaccccggt caagactttt ctttgaagga  
3351 tgactacaga cattaataaa tcgaagtaat tttgggtggg gagaagaggg  
3401 agattcaatt ttctttaacc agtctgaagt ttcatttatg atacaaaaga  
3451 agatgaaat ggaagtggca atataagggg atgaggaagg catgcctgga  
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-35-

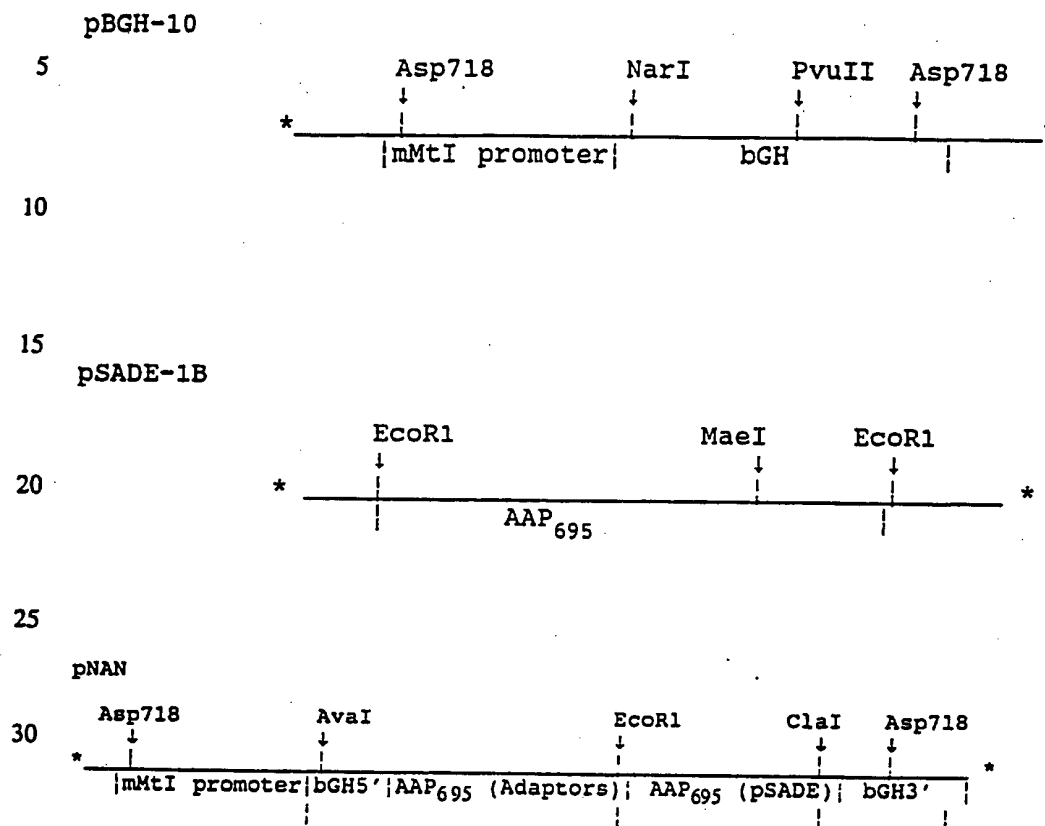
Chart 7

Chart 8

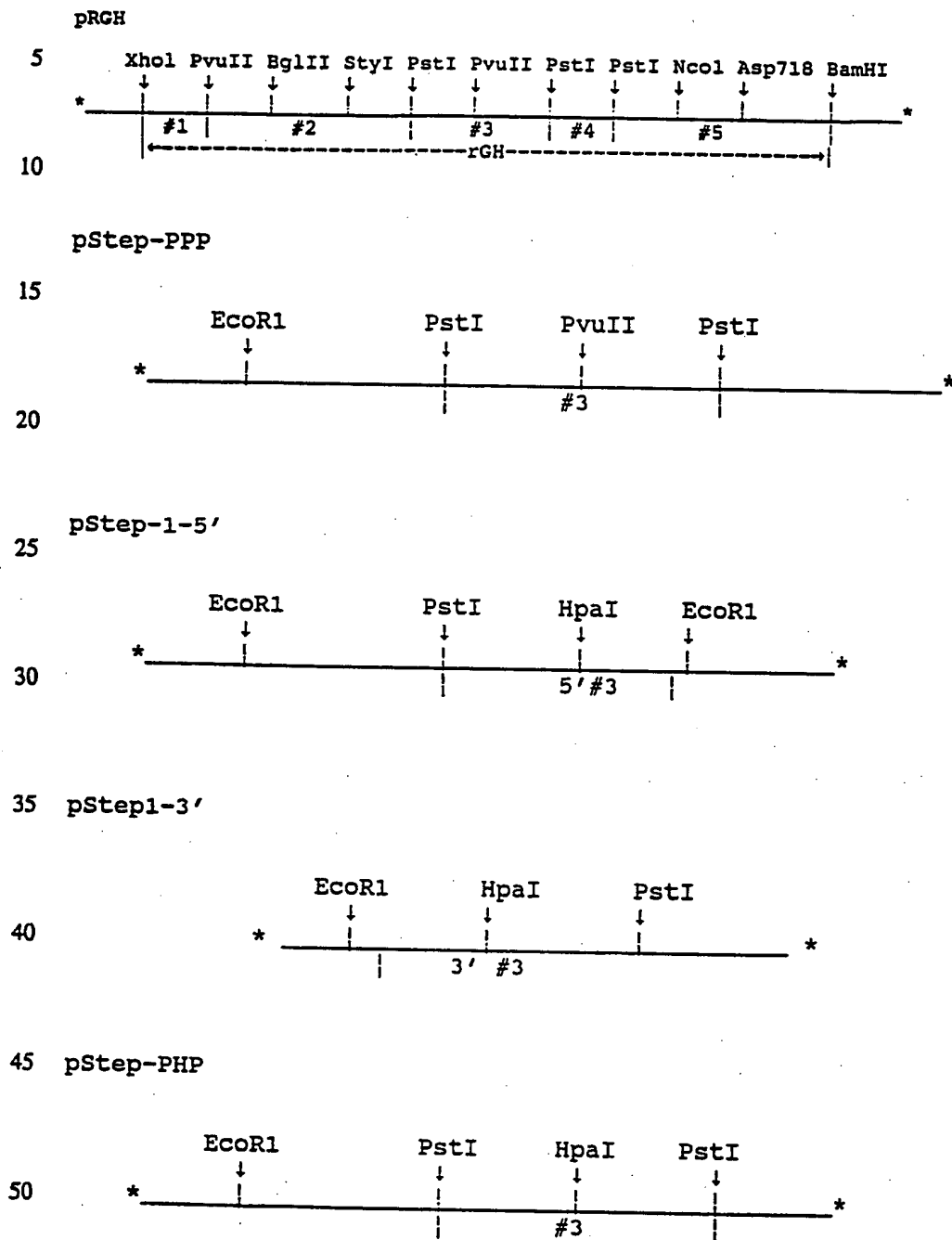


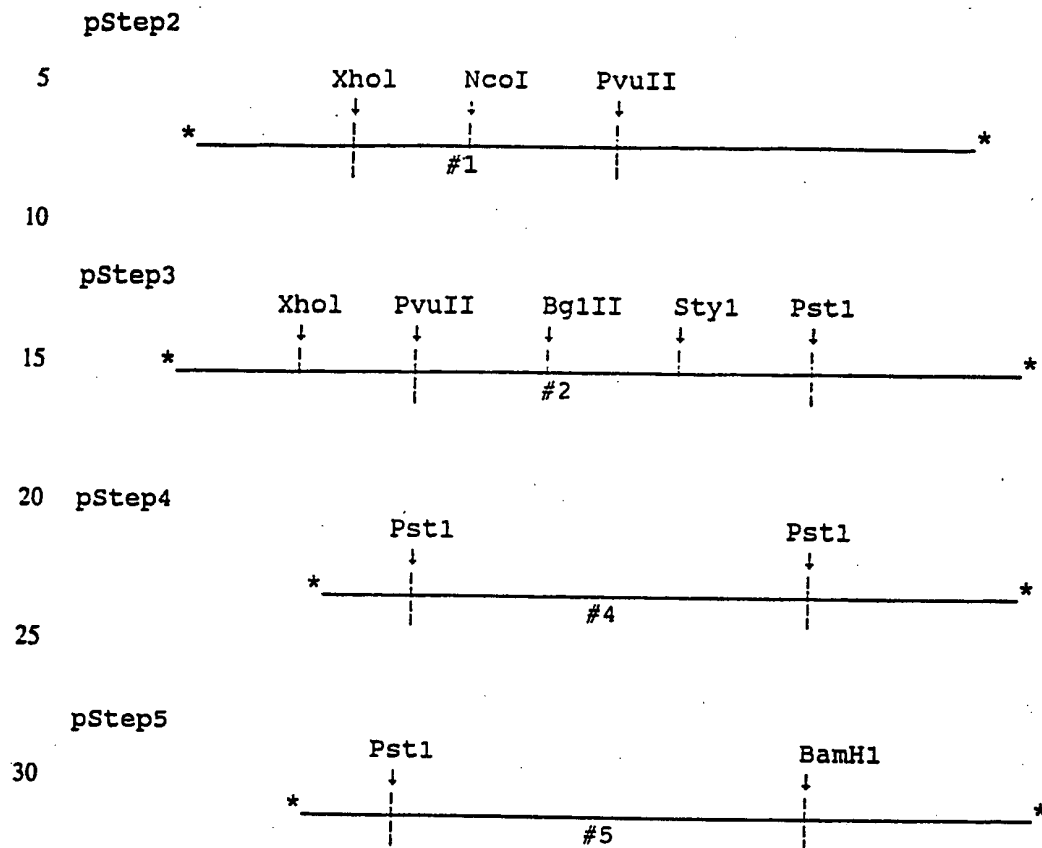
Chart 9

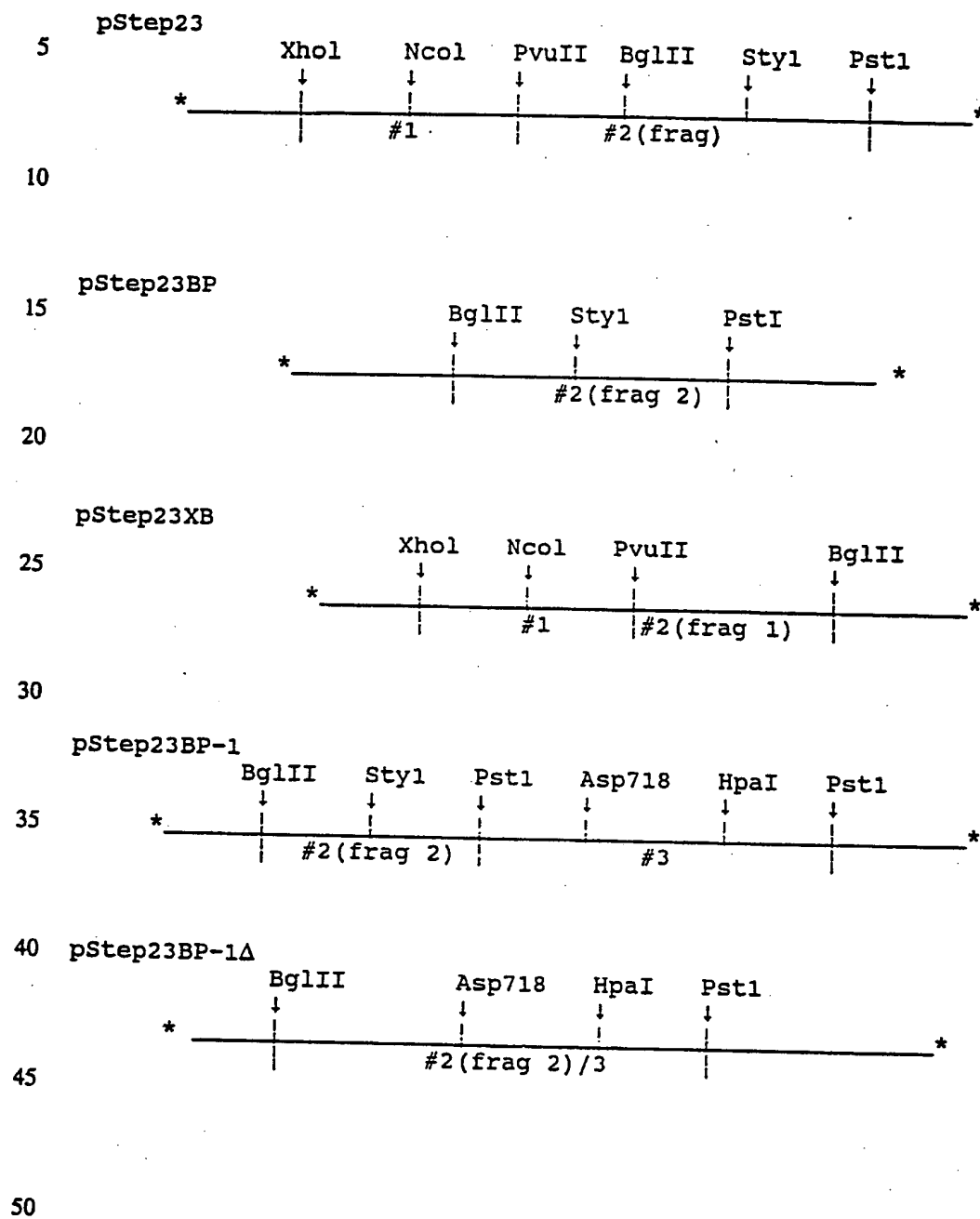
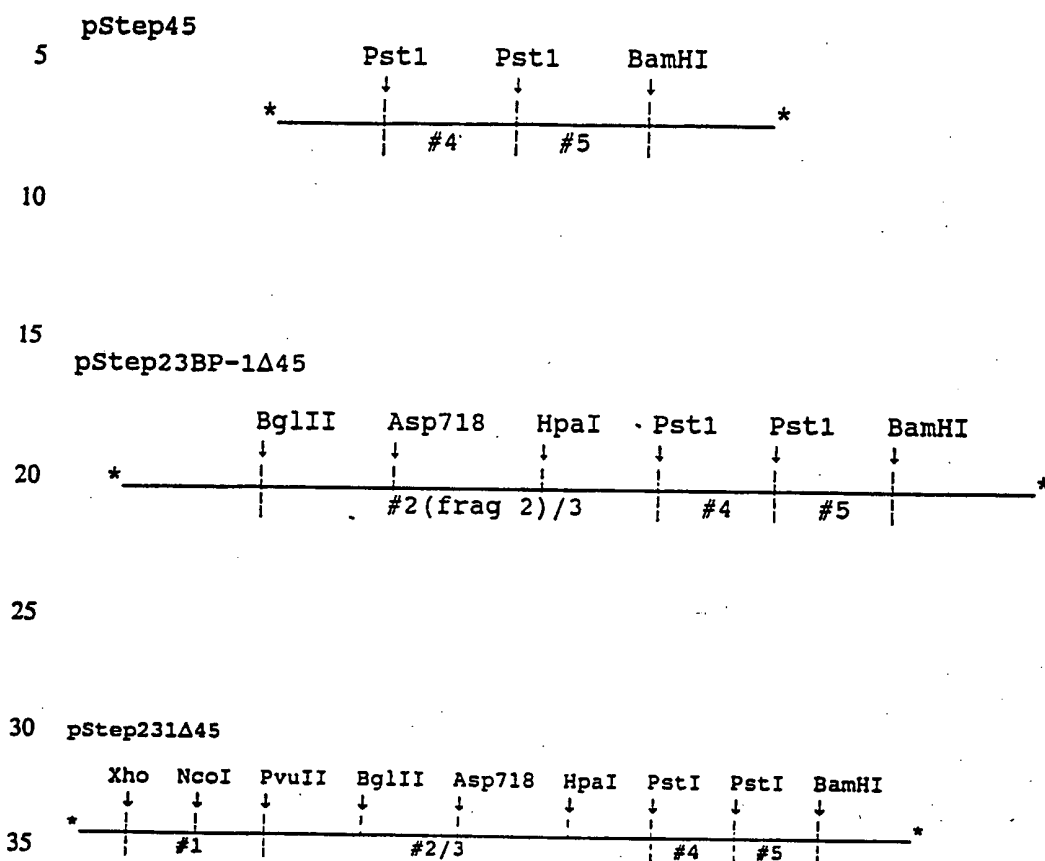
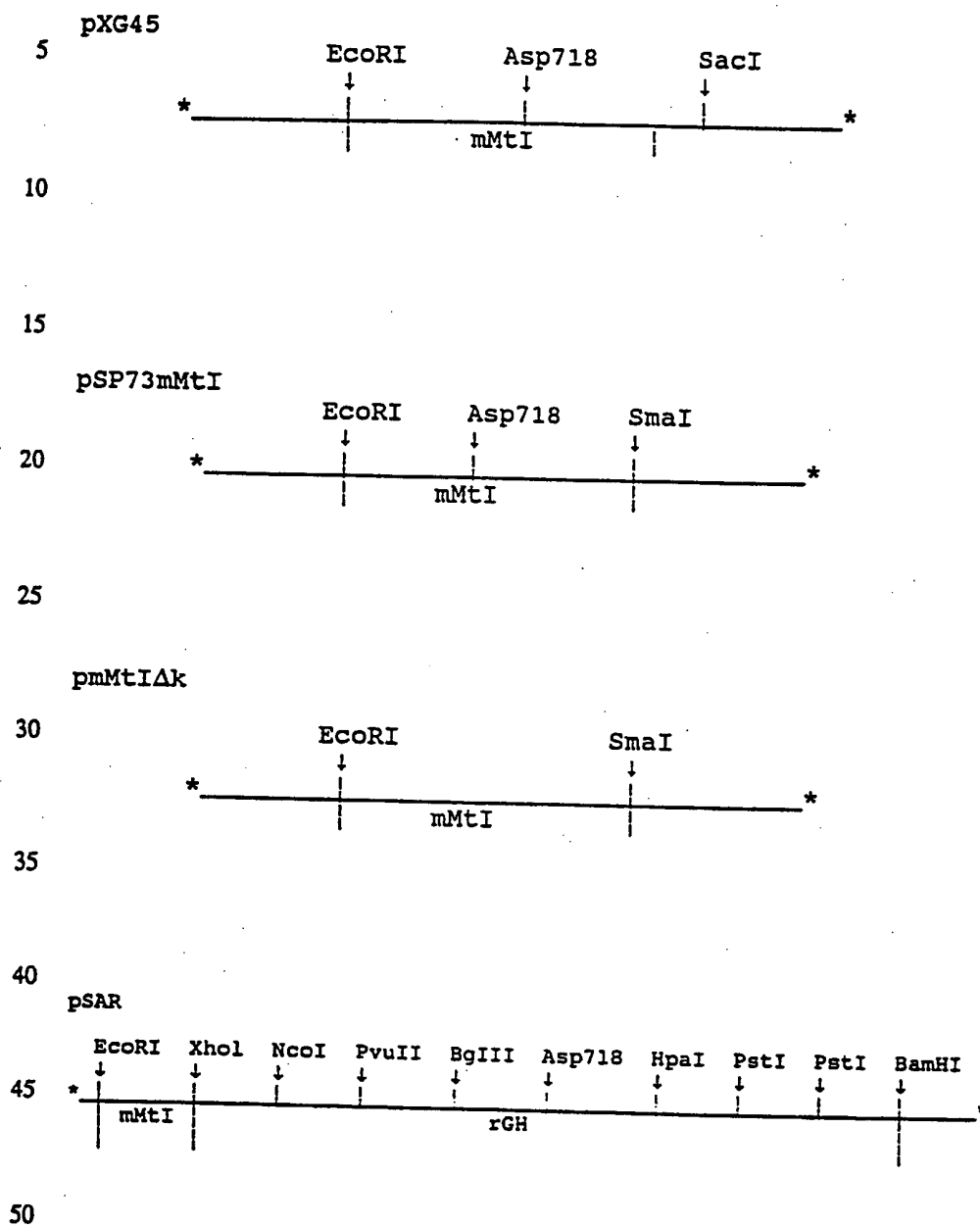
Chart 10



Chart 11

-40-


Chart 12

CLAIMS

1. A transgenic rodent comprising a mammalian metallothionein I (MtI) promoter operably linked to an Alzheimer amyloid precursor gene (AAP gene) operably linked to  
5 a mammalian growth hormone 3' untranslated region (GH 3'-UTR).
2. A transgenic rodent according to Claim 1 wherein said MtI promoter is a mouse metallothionein promoter (mMtI).
- 10 3. A transgenic rodent according to Claim 2 wherein said mammalian GH 3'-UTR is selected from the group comprised of mouse GH 3'-UTR (mGH 3'-UTR), rat GH 3'-UTR (rGH 3'-UTR), bovine GH3'-UTR (bGH3'-UTR) and human GH 3'-UTR (hGH 3'-UTR).
4. A transgenic rodent according to Claim 3 wherein said mammalian GH 3'-UTR is  
15 rGH 3'-UTR.
5. A transgenic rodent according to Claim 3 wherein said mammalian GH 3'-UTR is bGH 3'-UTR.
- 20 6. A transgenic rodent according to Claim 1 wherein said AAP cDNA is selected from the group consisting of AAP<sub>695</sub>, AAP<sub>751</sub>, and AAP<sub>770</sub>.
7. A transgenic rodent according to Claim 3 wherein said AAP cDNA is selected from the group consisting of AAP<sub>695</sub>, AAP<sub>751</sub>, and AAP<sub>770</sub>.  
25
8. A transgenic rodent according to Claim 7 wherein said AAP cDNA is AAP<sub>695</sub>.
9. A transgenic rodent according to Claim 8 wherein said mammalian GH3'-UTR is bGH 3'-UTR.  
30
10. A transgenic rodent according to Claim 8 wherein said mammalian GH-3'-UTR is rGH 3'-UTR.

11. A transgenic rodent according to Claim 1 further comprising DNA encoding of mammalian GH signal sequence.
12. A transgenic rodent according to Claim 11 wherein said AAP gene is AAP<sub>695</sub>.
- 5 13. A transgenic rodent according to Claim 12 wherein MtI promoter in mMtI, said mammalian GH-3'-UTR is bGH-3'-UTR and said DNA encoding a mammalian signal sequence is DNA encoding bGH signal sequence.
- 10 14. A transgenic rodent according to Claim 13 wherein said MtI promoter is mMtI, said mammalian GH-3'-UTR is rGH-3'-UTR and said DNA encoding a mammalian signal sequence is DNA encoding rGH signal sequence.
- 15 15. A transgenic rodent according to Claim 1 wherein said rodent is a mouse.
16. A recombinant DNA molecule comprising a metallothionein I (MtI) promoter operably linked to an Alzheimer amyloid precursor gene (AAP gene) operably linked to a mammalian growth hormone 3' untranslated region (GH 3'-UTR).
- 20 17. A recombinant DNA molecule according to Claim 16 wherein said MtI promoter is a mouse metallothionein I (mMtI) promoter.
18. A recombinant DNA molecule according to Claim 16 wherein said mammalian GH 3'-UTR is selected from the group comprised of mouse GH 3'-UTR (mGH 3'-UTR), rat
- 25 GH 3'-UTR (rGH 3'-UTR) bovine GH3'-UTR (bGH3'-UTR) and human GH 3'-UTR (hGH 3'-UTR).
19. A recombinant DNA molecule according to Claim 16 wherein said AAP gene is selected from the group consisting of AAP<sub>695</sub>, AAP<sub>751</sub>, and AAP<sub>770</sub>.
- 30 20. A recombinant DNA molecule according to Claim 16 further comprising DNA encoding of mammalian signal sequence.

## International Application No.

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>			
According to International Patent Classification (IPC) or to both National Classification and IPC			
Int.Cl. 5 C12N15/00; C12N15/12	A07K67/027;	C12N15/18;	C12N15/85
<b>II. FIELDS SEARCHED</b>			
Minimum Documentation Searched <sup>7</sup>			
Classification System	Classification Symbols		
Int.Cl. 5	C12N ;	C07K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>			
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>			
Category <sup>a</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	
P,A	EMBO JOURNAL. vol. 10, no. 2, February 1991, EYNHAM, OXFORD GB pages 289 - 296; WIRAK, D.O. ET AL.: 'REGULATORY REGION OF HUMAN AMYLOID PRECURSOR PROTEIN (APP) GENE PROMOTES NEURON-SPECIFIC GENE EXPRESSION IN THE CNS OF TRANSGENIC MICE' see the whole document	1	
P,Y	SCIENCE. vol. 253, no. 5017, 19 June 1991, LANCASTER, PA US pages 323 - 325; WIRAK, D.O. ETAL.: 'Deposits of amyloid beta protein in the Central Nervous System of transgenic mice' see the whole document	1-4,6-8, 10-12, 14-20,20	
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents :<sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>			
<b>IV. CERTIFICATION</b>			
Date of the Actual Completion of the International Search <div style="text-align: center; font-weight: bold;">19 FEBRUARY 1992</div>		Date of Mailing of this International Search Report <div style="text-align: center; font-weight: bold;">26. 02. 92</div>	
International Searching Authority <div style="text-align: center; font-weight: bold;">EUROPEAN PATENT OFFICE</div>		Signature of Authorized Officer <div style="text-align: center; font-weight: bold;">CHAM BONNET F.J.</div> <div style="text-align: right;">  </div>	

**III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)**

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,Y	<p>JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 266, no. 32, 15 November 1991, BALTIMORE US pages 21331 - 21334; SANDHU, F.A. ET AL.: 'Expression of the human beta-amyloid protein of Alzheimer's disease specifically in the brains of transgenic mice' see the whole document</p>	<p>1-4,6-8, 10-12, 14-20</p>
Y	<p>--- COLD SPRING HARBOR SYMPOSIA ON QUANTITATIVE BIOLOGY vol. L, 1985, COLD SPRING HARBOR, USA pages 389 - 387; EVANS, R.M. ET AL.: 'Inducible and developmental control of neuroendocrine genes' cited in the application see page 395 - page 396</p>	<p>1-4,6-8, 10-12, 14-20</p>
Y	<p>--- ANNUAL REVIEW OF NEUROSCIENCE vol. 11, 1988, PALO ALTO, USA pages 353 - 372; COWAN, W.M. ET AL.: 'Transgenic mice: applications to the study of the nervous system' see page 358, line 14 - line 21</p>	<p>1-4,6-8, 10-12, 14-20</p>
Y	<p>--- WO,A,8 906 689 (THE MCLEAN HOSPITAL CORPORATION) 27 July 1989 see the whole document</p>	<p>1-4,6-8, 10-12, 14-20</p>

US 9106727  
SA 52959

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